BLOOD CLOTTING AND ALLIED PROBLEMS

Transactions of The Second Conference
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Edited by

JOSEPH E FLYNN
DEPARTMENT OF PATHOLOGY
COLUMBIA UNIVERSITY
NEW YORK N Y

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PARTICIPANTS

DR IRVING S WRIGHT *Chairman*
Department of Medicine Cornell University Medical College
New York N Y

DR JOSEPH E FLYNN *Secretary*
Department of Pathology Columbia University
College of Physicians & Surgeons
New York N Y

DR NELSON W BARKER
Clinical Section Mayo Clinic
Rochester Minn

DR CHARLES H BEST
Department of Physiology Univ of Toronto Medical College
Toronto Canada

DR CHARLES E BRAMBEL
Mercy Hospital
Baltimore Md

DR KENNETH M BRINKHOUS
Department of Pathology Univ of North Carolina, School of Medicine
Chapel Hill N C

DR C LOCKARD CONLEY
Johns Hopkins Hospital
Baltimore Md

DR JOHN T EDSALL
Department of Physiological Chemistry Harvard Medical School
Boston Mass

DR JOHN H FERGUSON
Department of Physiology Univ of North Carolina School of Medicine
Chapel Hill N C

DR JOHN D FERRY
Department of Chemistry Univ of Wisconsin
Madison Wis

DR L B JAQUES
Department of Physiology Univ of Saskatchewan
Saskatoon Saskatchewan Canada

DR MELVIN H KNISELY
Department of Anatomy The Medical College of the State of South Carolina
Charleston S C

DR KARL PAUL LINK
Department of Biochemistry Univ of Wisconsin
Madison Wis

DR JOHN H OLWIN
Department of Surgery Univ of Illinois Medical School
Chicago Ill

DR RALPH S OVERMAN
Department of Medicine Cornell University Medical College
New York N Y

DR ARMAND J QUICK
Department of Biochemistry Marquette Univ School of Medicine
Milwaukee Wis

DR WALTER H SEEGERS
Department of Physiology Wayne Univ College of Medicine
Detroit Mich

DR H P SMITH
Department of Pathology Columbia Univ College of Physicians & Surgeons
New York N Y

DR L M TOCANTINS
Department of Medicine Jefferson Medical College
Philadelphia Pa

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Second Conference on Blood Clotting and Allied Problems

January 24-25, 1949

JOSIAH MACY, JR FOUNDATION CONFERENCE PROGRAM

FRANK FREMONT SMITH

Medical Director

With the accelerating rate at which new knowledge is accumulating and with the increasing recognition that nature is of one piece it becomes evident that the continued isolation of the several branches of science from one another is a serious obstacle to scientific progress

Nowhere in science is the need for combined operations more evident than in medicine Today to be effective medical research and practice must embrace data from all the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other for advances in one field are frequently dependent upon knowledge derived from quite another discipline

Although the fertility of the multi-discipline approach is thus recognized universities scientific societies and journals have not yet made adequate provision for channels of interdisciplinary communication

The Josiah Macy Jr Foundation therefore has endeavored to meet this need by bringing together for a series of two-day annual conferences a small group of investigators representing in so far as possible all the branches of science which bear on a chosen problem These round table discussions of research experience concepts and plans are conducted in a friendly and informal atmosphere which promotes communication cross-fertilization of ideas and cooperation The success of such an endeavor is dependent upon full participation of all members in the discussion Accordingly the attendance at any conference is limited to twenty five

In order to share with a wider group of investigators and students the essential quality of these conferences the informal nature and tempo of the discussions in so far as possible are preserved in the published transactions

SEPARATION AND ASSAY OF A LIPID ANTITHROMBOPLASTIN FROM HUMAN BRAIN, BLOOD, PLASMA AND PLASMA FRACTIONS *

L M TOCANTINS and R T CARROLL

Division of Hematology Department of Medicine Jefferson Medical College and Hospital

It is our intention at this time to present to the Conference a condensed account of our attempts to separate and study a lipid antithromboplastin from extracts of human brain blood plasma and plasma fractions

Human Brain Extracts The essential steps in the method of extraction at present are as follows [Tocantins L M Carroll R T and McBride T J *Proc Soc Exp Biol and Med* 68 *110 (1948)*] 30 grams of acetone dried brain powder are extracted for 5 6 days at 5°C with 600 ml of absolute methanol The super natant is filtered off and the filtrate distilled in vacuo at 40 C The residue remaining in the flask after the distillation is com pleted is removed with absolute ethyl ether The ether solution is kept at 5 C overnight during which time a precipitate settles out The supernatant is decanted off and the precipitate washed with cold ether This is kept at 5 C overnight and the supernatant again removed The combined filtered clear supernatant ether extracts are evaporated off leaving a creamy white waxy residue Suspensions of this material are made in 0 85% NaCl the pH being adjusted to 7 0 7 3 with dilute NaOH

A suspension of the inhibitor thus prepared has an immediate and progressive antithromboplastin and anticephalin action It has no immediate or progressive antithrombin activity and does not delay the clotting of fibrinogen by thrombin indeed it seems to accelerate it This enhancement of thrombin action on fibrinogen is now under study It seems analogous to that reported by Ware Fahey and Seegers [*Am Jour Physiol* 154 *140 (1948)*] for a factor present in an aqueous extract of platelets We have re garded this enhancement of thrombin action as due to an impurity

* Aided by a grant from the U S Public Health Service

in our preparations which we hope to remove, since it obviously offsets to a certain extent, the action of the anticoagulant on the pre-thrombin phases of clotting

Though there is much evidence that the lipid anticoagulant acts as an antithromboplastin [*Proc Soc Exp Biol, and Med*, 68, *110 (1948)*], this may not be regarded as conclusive since no tests with purified reagents have been performed The effectiveness of the lipid inhibitor decreases greatly if the blood or plasma to which it is added, has been allowed to undergo changes (after contact with glass tissue juices) before it is citrated Moreover if the inhibitor is added to the plasma after recalcification its activity is correspondingly diminished (Table 1) The human inhibitor exerts its greatest effect against human thromboplastin in human plasma This species preferential action which is manifested when thromboplastin of different species is used against human inhibitor [*Proc Soc Exp Biol and Med* 68 *110 (1948)*] seems additional evidence that the anticoagulant acts on the thromboplastin The clot accelerating action of solutions of Russell viper venom, which owe their potency to their ability to convert prothrombin to thrombin is not affected by the anticoagulant This seems to make it unlikely that the anticoagulant is an antiprothrombin The

TABLE 1

EFFECT OF ADDING THE BRAIN LIPID INHIBITOR TO NORMAL CITRATED PLASMA BEFORE AND AFTER RECALCIFICATION

	Clotting Time (seconds)
No inhibitor	536 *
Inhibitor added **	
1 Before recalcification	1867
2. 15 seconds after recalcification	1572
3 30 seconds after recalcification	1235
4 60 seconds after recalcification	848
5 120 seconds after recalcification	585

* 0 1 ml plasma 0 1 ml 0 85% NaCl 0 1 ml 0 02 M $CaCl_2$

** 0 1 ml plasma 0 1 ml 0.5% inhibitor 0 1 ml 0 02 M CaCl

(Inhibitor added *after* recalcification in all tubes except the first one)

Clotting time recorded from the moment Ca was added.

antithromboplastin effect of the inhibitor is probably not due to a direct binding or neutralizing action which it may, itself, have on thromboplastin It appears more likely that the lipid anti coagulant either (a) requires a plasma cofactor to become an *effective antithromboplastin or (b) it competes with thrombo*plastin for a substance (Ac globulin prothrombin?) required for the rapid formation of thrombin thus in effect, reducing the prothrombin conversion accelerating effect of thromboplastin

The anticoagulant is soluble in most of the lipid solvents has a waxy appearance and a rancid odor and its strongest suspensions give negative tests for protein and carbohydrate Our first trials led us to think that the inhibitor was heat labile and that tem peratures of 70 C or above for ten minutes impaired its activity It now seems that the reduction in activity is not due to the effect of heat on the inhibitor itself but on its degree of dispersion in the medium in which it is suspended If a well homogenized suspension of the anticoagulant is heated at 70 C for 5 minutes in a narrow tube and little evaporation results there is no significant loss in activity When heated in an open vessel with a wide surface exposed to evaporation there is a clear reduction in activity *if the lost volume of fluid is replaced and the suspension re*homogenized most of the potency it had before heating is restored It seems therefore that condensation of particles with consequent reduction in their active surface is responsible for the loss of activity previously reported as a result of heating (Table 2)

Homogenization The effect of homogenizing suspensions of *inhibitor in 0 85% NaCl using an electrically driven homogenizer* (Logeman) is shown on Table 3 This homogenizer operates at a stroke rate of 332 strokes per minute continually driving the suspension at high pressure through a narrow opening within a closed circuit The activity of the suspension depends to a certain extent on the duration of homogenization after a given period further homogenization produces little or no increase in potency *Concentrated suspensions require a longer period of homogenization* to reach a maximum effect than dilute ones The latter when well homogenized lose their milky appearance become translucent and mix readily with the plasma Further breakdown of the lipid particles to submicroscopic size (e g by exposure to ultrasonic waves) should enhance the potency of the suspension even further

TABLE 2.

EFFECT OF HEAT AND HOMOGENIZATION ON THE POTENCY OF THE BRAIN LIPID ANTICOAGULANT *

TYPE OF PREPARATION OF ANTICOAGULANT	MGM OF ANTICOAGULANT PER 0 1 ML. PLASMA		
	2	0 5	0 2
	CLOTTING TIME IN SECONDS		
1 Homogenized Suspension	93	35	18
2 Same as (1) Heated 70 —80 C 15 Minutes **	35	21	14
3 Same as (2) Homogenized 5 times after heating	82	29	17

* 0 1 ml of lipid anticoagulant 0 1 ml plasma 0 1 ml brain thromboplastin 0 1 ml 0 02 m $CaCl_2$ Control clotting time (without anticoagulant) 13 seconds

** 16% water loss during heating restored by adding H_2O gentle mixing no homogenization

As previously reported, the antithromboplastin activity of plasma is reduced by heating to 65 C for 5 minutes [Tocantins L M *Am Jour Physiol* 139 *265 (1943)*] Whether this change is due to interference with the degree of dispersion of the lipid in the plasma is not possible to state homogenization of the plasma after heating is impractical It is possible that in the blood or plasma and perhaps in certain tissues the lipid antithromboplastin is conjugated with a protein in a manner analogous to the relation of cephalin to the thromboplastic lipoprotein In such a conjugated form the anticoagulant might be (like the thromboplastic lipoprotein) heat labile while (like cephalin) when divorced from the protein it would be relatively heat stable Its solubility in water may be influenced likewise by whether it is in the free form or

as a lipoprotein complex, the free lipid is poorly soluble in water while its protein conjugate would probably be more soluble

TABLE 3

EFFECT OF DURATION OF HOMOGENIZATION ON THE POTENCY OF LIPID ANTITHROMBOPLASTIN SUSPENSIONS FROM TWO SOURCES

Duration of Homogenization	CLOTTING TIME (secs) OF ACTIVATED PLASMA* CONTAINING			
	Brain Antithrombopl		Blood Antithrombopl	0 85% NaCL
(Seconds)	1% Susp	6% Susp	0 5% Suspension	
0	30	129	22	14
15	77	174	25	
30	82	188	27	
60	91	216	28	
120	115	247	28	
240		289	28	
480	112	285	28	

* 0 1 ml. thromboplastin, 0 1 ml. 0 02 M CaCl 0 1 ml. inhibitor suspension (or 0 85% NaCl) 0 1 ml plasma Collodion Tubes 38 C

Standard of Antithromboplastin Activity In order to assay antithromboplastin activity of extracts obtained from various sources a standard of reference became necessary Since no anti coagulant with properties and mode of action similar to ours was available to be used as a standard it was decided to provide one by selecting a purified lot of the brain tissue inhibitor granting an arbitrary number of units to each mgm of the material in that lot and measure the activity of future lots by comparing them with that of 2-5 per cent suspensions of the standard lot Figure 1

illustrates the curve of activity of this standard anticoagulant at different concentrations Curves A and B represent tests done with the same standard, but on two successive days using a different plasma and thromboplastin Since the inhibitor is assayed in an activated clotting system (0 1 ml human thromboplastin, 0 1 ml 0 02 M $CaCl_2$, 0 1 ml plasma) which give a clotting time between 12 and 15 seconds in collodion tubes, a curve of activity of a well homogenized standard inhibitor must be worked out every time the potency of a given lot is to be measured This is to make allowance for slight differences in potency of the thromboplastin and the reactivity of the plasma on any particular day Since the concentration/activity ratio even when plotted on a logarithmic scale is not linear throughout, only the section of the curve between 30 and 200 seconds has been used for purposes of interpolation If the times obtained with the unknown fell outside these limits the strength of the unknown suspension was changed so that its time

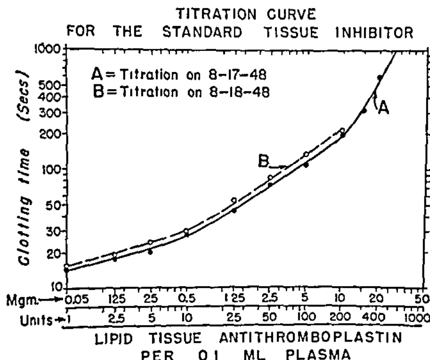


Figure 1 Curve of activity of the brain tissue lipid inhibitor used as a standard for assay

could fall within the useful limits of the curve The standard lot when kept at a temperature of –10 C did not seem to change significantly in potency for at least three months As further lots were purified and their potency established on the basis of the first standard they themselves were used as standards

Activity Curves of Coagulant Anticoagulant Mixtures A study of the behavior of tissue extracts when tested at various concentrations on properly collected stable plasma in collodion tubes disclosed that such extracts consist of a mixture of clotting inhibitors and accelerators the predominance of one group over the other will determine the shape of the activity curves of a given extract [Tocantins L M and Carroll R T, *Proc Soc Exp Biol and Med* 69 *431 (1948)*] An extension of this concept was made to the study of complex mixtures such as blood plasma and certain plasma fractions separated by the Cohn method

Figure 2 illustrates the changes in the rate of coagulation of normal and hemophilic blood in glass and collodion tubes after dilution with 0 85% NaCl The clot accelerating effect of dilution may be observed in either glass or collodion surfaces when hemophilic blood is used When normal blood is used dilution often tends to prolong the rate of coagulation in glass tubes while in collodion tubes a shortening almost always occurs These changes can be observed only when the blood has been collected with special precautions with oiled syringes a needle of large caliber (18 gauge) a clean rapid venipuncture the blood s being aspirated without air bubbles and the proper amounts being transferred without delay to tubes where the correct quantity of 0 85% NaCl has been placed If difficulty is experienced in collection necessitating repeated punctures of the vein the undiluted blood becomes hypercoagulable and dilution will only serve to prolong the rate of coagulation even in collodion tubes Likewise hypercoagulable blood obtained from individuals a few hours after severe hemorrhage will even if carefully collected yield more prolonged rates of coagulation on dilution [*Proc Soc Exp Biol and Med* 69 *431 (1948)*] Since after a severe hemorrhage dilution of the blood with tissue fluid occurs the hypercoagulability may be either due to simple dilution of the blood or entrance into it of clot accelerating substances from the tissue fluids In any event dilution seems to upset the balance between anticoagulants and procoagulants in the blood [Tocantins L M *Blood* 1 *156 (1946)*] Since in normal

EFFECT OF DILUTION (0.85 % NaCl) ON THE COAGULATION OF HEMOPHILIC BLOOD USING TWO TYPES OF SURFACE.

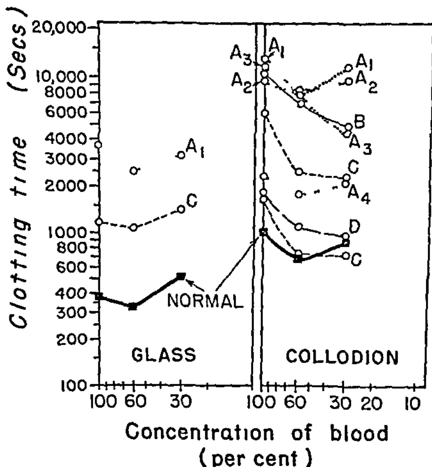


Figure 2 Each letter accompanying a curve designates a different individual and the subscript indicates the number of the specimen of blood collected from that subject. The curve for normal blood is the mean of 21 determinations on 14 normal subjects Hemophilic blood C which had only a slightly delayed rate of coagulation in glass tubes yielded a dilution curve essentially like normal blood in glass while the blood of subject A_1 with a longer clotting time displayed a curve of dilution in glass tubes essentially like that in collodion tubes Three concentrations of blood used 100% (1 ml blood) 60% (0 6 ml blood 0 4 ml 0.85% NaCl) 30% (0.3 ml blood 0 7 ml 0 85 NaCl) For further technical details consult [*Proc Soc Exp Biol and Med* 69 *431 (1948)*]

blood an excess of procoagulants (platelets, prothrombin Ac globulin fibrinogen) over the amount needed for rapid and effective clotting already exists dilution by reducing the anticoagulants enhances the action of procoagulants. The *direction* and *extent* of the change in the rate of coagulation after dilution serves to indicate whether in a given specimen of blood the anticoagulants or the procoagulants are in dominance The effect of dilution on hemophilic blood seems to indicate that this type of blood is a *predominantly* anticoagulant mixture while dilution of post-hemorrhagic blood characterizes it as a *predominantly* procoagulant mixture Significantly heparinized blood behaves on dilution like a predominantly anticoagulant mixture (Figure 3) its dilution curves resembling those of hemophilic blood

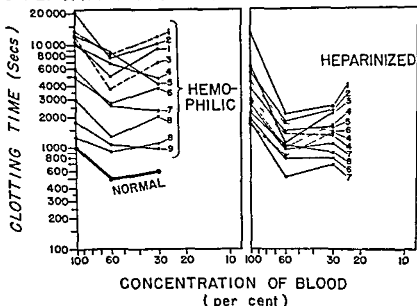


Figure 3 The numbers designate individual subjects Heparinized blood originated from patients receiving heparin because of thrombo embolic disease Collodion tubes 38 C Plasma prothrombin by the one stage method, varied between 90 and 120 per cent normal

Method of Extraction of the Lipid Antithromboplastin from Blood Venous blood is removed by direct puncture of an engored vein through a polyethylene coated 16 gauge needle and allowed to flow into a wide-mouthed vessel containing 1000 ml of cold absolute methanol (C P Baker's Analysed) (Technical Grade not suitable) The blood should enter the alcohol directly, without contact with the glass sides of the container The vessel is gently swirled during the collection, the blood being withdrawn until the combined volume of the alcohol and blood reaches a previously measured mark The withdrawal should be at a rate no slower than 1 cc per second The final mixture consists of fine reddish granules settling through a light yellow liquid There should be little or no hemolysis The mixture is allowed to extract in the cold for 5 7 days with occasional swirling, it is then filtered through two layers of filter paper and distilled at 40 C The aqueous residue remaining in the distilling flask is removed with absolute ethyl ether This is allowed to extract with the same ether, in the cold, with occasional shaking for 3 5 days The layers are then separated by means of a separatory funnel The aqueous layer is discarded The ether layer is evaporated *in vacuo* and the residue is treated with C P acetone at room temperature for two hours The acetone solution contains a coagulant and is treated separately The acetone insoluble ether residue is weighed and suspended in 0 85% NaCl homogenized in a Logeman homogenizer and its pH adjusted

The physiochemical characteristics of this material are as follows it is a dark brown waxy substance easily suspended in 0 85% NaCl and water and having a slightly rancid odor It is soluble in ether, methanol carbon tetrachloride and chloroform and insoluble in acetone Like the tissue lipid antithromboplastin it stands temperatures of 80 C for ten minutes without significant loss of activity if rehomogenized subsequent to the heating Its potency is not affected by prolonged dialysis (72 hours against cold running water) Unlike the brain inhibitor its solutions are brown and at 0 5% concentration it has a pH of 8 4 It gives negative tests for protein carbohydrate and sulfur Its anticoagulant action seems directed against thromboplastin It does not bind calcium nor does it have an antithrombin or antifibrinogen action It is not neutralized by protamine Like the brain tissue inhibitor it enhances the action of thrombin on fibrinogen and is impotent against the clot accelerating action of Russell viper venom.

Extraction of Liquid Plasma Method Blood is aspirated through a 16 gauge needle coated with polyethylene into a syringe containing 3 8% Trisodium citrate (1 part citrate 9 parts blood) The citrated blood is placed in paraffin coated tubes and centrifuged at 3000 r p m for one hour The upper two-thirds of the plasma is removed with paraffin coated droppers and added slowly to cold absolute methanol (50 ml plasma 500 ml methanol C P Baker's Analysed) The mixture is allowed to extract in the cold for 5 7 days with occasional gentle swirling At the end of this period the mixture is filtered and the filtrate distilled at 40 C The subsequent steps are the same as described for the extraction of the blood antithromboplastin The pH of a 3 per cent suspension in 0 85% NaCl was about 9 0 before adjustment

The physiochemical characteristics of the lipid antithromboplastin extracted from plasma are except for the color essentially similar to those of the antithromboplastin obtained from blood It is light yellow waxy fairly easily suspended in 0 85% NaCl and exerts the same action on activated plasma as the extracts from blood and brain tissue As will be shown later the yield and potency of extracts obtained from liquid plasma were lower than that from any other source

Plasma Fractions A study of the effect on the rate of coagulation of stable plasma of the addition to it of various plasma fractions separated by the cold methanol method of Cohn (fractions obtained through the courtesy of Dr Edwin Cohn of the Harvard Medical School and Dr Robert Pennell of the Biological Laboratories of Sharp and Dohme) disclosed that fractions IV 1 and IV-3 4 had biphasic curves of activity This was taken to indicate that these two fractions were complex procoagulant-anticoagulant mixtures [*Proc Soc Exp Biol and Med* 69 *431 (1948)*]

Extraction of Plasma Fractions Method To 20 grams of the dried plasma fraction is added 400 ml of absolute methanol (C P Baker's Analysed) The pH of the solution is maintained at 6 0 6 5 and the mixture is allowed to extract for 5 7 days at 5 C with occasional swirling The supernatant is filtered the filtrate distilled at 40 C and the residue removed from the distilling flask with absolute ethyl ether The ether solution is allowed to remain at 5 C overnight and the clear supernatant carefully removed If desired additional crops may be obtained by adding fresh ether to the white insoluble cold residue and allowing the mixture to extract

overnight The combined clear ether solutions are evaporated *in vacuo* and the resulting residue treated for one hour with C P acetone The acetone solution may contain a coagulant and is handled separately The acetone insoluble ether residue is weighed and it is then ready to be mixed with 0 85% NaCl homogenized and have its pH adjusted to 7 0 7 3 with dilute NaOH

The physiochemical characteristics and mode of action of the anticoagulant material extracted by this method were essentially like those described for the brain and plasma extracts

TABLE 4

COMPARISON OF YIELD OF ANTITHROMBOPLASTIN UNITS (A U) FROM EXTRACTS OF NORMAL AND HEMOPHILIC BLOOD

	EXPERIMENT NO				MEAN
	1	2	3	4	
Amount of blood collected (ml)					
Normal	50	185	120	100	113 7
Hemophilic	50	120	110	100	95
Yield of Extract (mgm)					
Normal	78	85	120	40	80 7
Hemophilic	108	95	135	85	105 7
A U per mgm Extract					
Normal	9 8	13 8	3 5	10	9 2
Hemophilic	40	56	50	26	43 0
A U per ml Blood					
Normal	15 2	6 5	3 5	4 0	7 3
Hemophilic	86 4	44 3	70 4	22 1	55 8

Assay of Activity of Extracts from Normal and Hemophilic Blood A comparative study of the antithromboplastin content of normal and hemophilic blood was carried out as follows blood was collected in groups of paired subjects one a known hemophilic and the other a normal each collection being timed so that no more than a few minutes elapsed between collections from the two subjects The bloods were then processed as previously described At every step the two bloods were handled alike and eventually they were tested almost simultaneously against the same activated plasma system. The distillation and separation were carried out so as to prevent any loss of the extracted materials Three per cent suspensions of each extract were prepared pH adjusted homogenized with a hand homogenizer the same number of times and assayed for antithromboplastin potency against the standard lipid brain inhibitor The results of four paired assays are given on Table 4 Though slightly less blood was collected from the hemophilic group the mean of antithromboplastin units per ml of collected blood was over 7 times higher in this group The yield of the extract was somewhat higher in the hemophilic and the units of antithromboplastin per mgm of the extract were over 4 times higher in hemophilic blood (range 2½ to 16)

Comparison of the Yield of Antithromboplastin from Normal and Hemophilic Plasma. Normal and hemophilic plasma were collected from the respective donors in pairs (one normal and one hemophilic) and processed as described above In addition native plasma was separated from hemophilic blood collected in chilled paraffin tubes and centrifuged at 5 C for one hour at 5000 r p m The three samples of plasma namely normal citrated plasma hemophilic citrated plasma and the hemophilic native plasma were processed at the same time three per cent suspensions made of the extracts and their potency assayed against the standard human brain inhibitor As shown in Table 5 from 2 to 4 times as much antithromboplastin was extracted from hemophilic citrated or hemophilic native plasma as from normal plasma The gross yield of the extract from hemophilic plasma was proportionately higher In contrast with the yields from whole blood the antithromboplastin units per mgm. of extract were essentially the same in the three different types of plasma (0 3 A U per mgm.) With liquid plasma it would appear that the higher yield of extractable material accounts for the greater number of antithromboplastin units in the hemophilic plasma While hemophilic plasma yielded a little

over 4 A U per ml, hemophilic blood yielded over 13 times as much namely 55 8 units The difference between the yields of normal plasma against normal blood is also of a seven fold order (1 unit per ml of normal plasma against 7 3 units per ml of blood) This may mean that some, if not most of the antithrombo plastin of the blood is trapped with or adsorbed on the red cells and is centrifuged down with them when the plasma is separated

TABLE 5

COMPARISON OF YIELDS OF ANTITHROMBOPLASTIN UNITS (A U) EXTRACTED FROM NORMAL AND HEMOPHILIC PLASMA

	Normal Citrated Plasma		Hemophilic			
			Citrated		Native	
	No 1	No 2	No 1	No 2	No 1	No 2
Amount of Plasma Collected (ml)	47 0	48 0	47 0	47 0	44 4	43 6
Total A U	55 5	43 5	155 4	218 4	200	104 7
Yield of Extract in mgm	185	145	518.	726	500	349
A U per mgm of Extract	0 3	0 3	0 3	0 3	0 4	0 3
A U per ml Plasma	1 18	0 9	3 3	4 6	4 5	2 4
A. U per mgm. Dry Plasma *	0 016	0 012	0 047	0 066	0 066	0 034

* Calculated on the basis that 100 ml plasma contains 7 gms dry solids

Assay of Activity of Extracts of Plasma Fractions Concentration/activity tests on solutions of the various plasma fractions led us to believe that most of the anticoagulant would probably be

found in fractions IV 1 and IV 3, 4 These fractions also display the greatest degree of anticephalin activity On Table 6 are shown the effect of incubation of solutions of two lots of fractions I IV 1 and

TABLE 6

ANTICEPHALIN AND ANTITHROMBOPLASTIN ACTIVITIES OF TWO LOTS OF THREE PLASMA FRACTIONS

Lot	Fraction I		Fraction IV 1		Fraction IV-3 4	
	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
1 Anticephalin Activity *						
0' Incub (secs)	85	95	74	110	82	224
30' Incub (secs)	76	102	93	648	287	1344
1a Control **						
0' Incub (secs)	232	107	244	107	144	107
30' Incub (secs)	241	112	257	112	147	112
2. Antithrombopl. Activity *						
0' Incub (secs)	81	94	83	97	69	142
30' Incub (secs)	72	101	87	132	84	232
2a. Control **						
0' Incub (secs.)	220	98	384	98	241	98
30' Incub (secs)	229	114	398	114	247	114
pH (before adjustment)	7 02	6 8	4 96	5 57	5 65	4 91

* 0 1 ml 3% plasma fraction 0 1 ml cephalin (or dilute thromboplastin) (0 or 30 mins incubation) 0 1 ml. plasma 0 1 ml. $CaCl_2$ (variable molar concentration)

** 0 1 ml 0 85% NaCl 0 1 ml cephalin (or dil. thrombopl.) (0 or 30 mins incub) 0 1 ml plas 0 1 ml 0 02 M $CaCl_2$

IV 3, 4 with human brain thromboplastin or cephalin on the clotting time of the mixture Lot 2 of fraction IV-3 4 displayed the greatest activity both against cephalin and brain thromboplastin (aqueous brain tissue extract) Fraction I (Table 6) and fractions II III and V had no significant activity The results of these tests as well as of the extractions (Table 7) suggest that the content of extractable lipid inhibitor varies in different lots of the same plasma fraction This may be due to differences in the method of fractionation or what is more likely, to differences in the mode of collection, storage and handling of the plasma from which the fractions are separated

TABLE 7

YIELD OF ANTITHROMBOPLASTIN FROM EXTRACTS OF TWO LOTS OF PLASMA FRACTIONS

Lot	Fraction	Amt. (gms)	Yield of Extr (mgm)	Units per mgm	Total Anti throm bopl Units	Units per gm of Dry Fraction
1	I	20	154	0	0	0
1	III 1	20	1300	0 1	130	6 5
1	IV 1	20	360	3 0	1080	54
1	IV 3 4	20	330	29	9570	478 5
1	V	20	182	0 2	36 4	1 8
2	I	20	55	0	0	0
2	III 1	20	804	0 4	321	16 05
2	IV 1	20	658	7	4706	235 3
2	IV 3 4	20	920	8 8	8096	404 8
2	V	20	91	0 2	18 2	0 92

On Table 7 is given the yield of antithromboplastin from extracts of 2 lots of plasma fractions Most of the activity is found in fraction IV-3 4 No active material was recovered from fraction I Fraction III 1 had a slight amount and a trace of activity was found in fraction V A good direct correlation was obtained between the distribution of anticephalin activity and the yield in anticoagulant units recovered from each fraction.

Even though most of the inhibitor activity was extracted from fraction IV-3 4 the lipid inhibitor extracted from brain tissue exerted its greatest effect against fraction I On Table 8 is shown the effect (with and without incubation) of this inhibitor on the clot accelerating action of the various plasma fractions The brain lipid inhibitor has its greatest immediate and progressive anticoagulant effect on fraction I and least on fraction V It would seem then that on fraction I (known to carry plasma thromboplastin) are concentrated the natural antagonists to the lipid antithromboplastin.

Wright Discussion of Dr Tocantins presentation will be deferred until Dr Overman gives his data

TABLE 8

IMMEDIATE AND PROGRESSIVE ANTICOAGULANT ACTIVITY OF THE TISSUE INHIBITOR ON COHN PLASMA FRACTIONS

3% Fraction	0 85% NaCl (ml)	Lipid Tissue Inhibitor (mgm)	Plasma (ml)	$CaCl_2$ (ml)	Clotting Time (secs)*		Inhibitor Activity**	
					0' incub	30 incub	0' incub	30 incub
	0 2		0 1	0 1	412			
	0 1	1 0	0 1	0 1	3138	3152	7 5	
I	0 1		0 1	0 1	66	64		
I		1 0	0 1	0 1	1024	1533	18	23
II III	0 1		0 1	0 1	56	57		
II III		1 0	0 1	0 1	510	410	9 1	7 1
IV 1	0 1		0 1	0 1	70	71		
IV 1		1 0	0 1	0 1	530	575	7 5	8
IV 3 4	0 1		0 1	0 1	89	87		
IV 3 4		1 0	0 1	0 1	640	765	7 1	8 6
V	0 1		0 1	0 1	404	390		
V		1 0	0 1	0 1	1110	1116	2 7	2.8

* 0 1 ml fraction (or 0 85% NaCl) + 0 1 ml 1% inhibitor solution (or 0 85% NaCl) (0 or 30 minutes incubation) + 0 1 ml. plasma + 0 1 ml. $CaCl_2$ (optimum molar concentration adjusted for each fraction) Fractions dissolved in H_2O pH adjusted to 7 3—7 4

** Expressed as the extent (multiples) to which clotting was delayed by the lipid inhibitor

THE CHEMICAL PURIFICATION AND MODE OF ACTION OF A THROMBOPLASTIC INHIBITOR*

RALPH S OVERMAN

Department of Medicine Cornell University Medical College

In our work on the isolation and chemical purification of thromboplastin we isolated a fraction from active rabbit brain and also from lung thromboplastin which inhibited the action of thromboplastin as measured by the prothrombin clotting time This material also prolonged the coagulation of whole blood [Overman R S *Macy Foundation Conference on Blood Clotting and Allied Problems* 1 *144 147 (1948)* Overman R S & Wright I S *J Biol Chem* 174 *759 (1948)*] Fractions with similar activity were also isolated from soybean phosphatide ' asolectin (kindly supplied by Dr A Scharf of Associated Concentrates Inc Woodside Long Island New York) and from normal human plasma The inositol phosphatide fraction of brain lung soybean and plasma phosphatides gave us our most active preparations

As our work on the further purification and investigation of the chemical structure of the phosphatide inhibitor progressed we thought it advisable to determine what had happened to the thromboplastic activity of our starting material The fraction remaining after removing the phosphatide inhibitor exhibited absolutely no thromboplastic activity If thromboplastin or any material with potential thromboplastic activity was present in the residue we were unable to activate it by any simple chemical or physical means Therefore we explored the possibility that something in the residue was associated with the inhibitor which we had removed the two acting together to produce thromboplastic activity On fractionation of this residue we obtained a material presumably protein in nature which we designate as thrombo-protein or for simplification alpha This fraction was added to plasma in a constant amount When the phosphatide inhibitory fraction (designated as I) is added in increasing amounts to this system and prothrombin times determined the curve shown in Figure 4 was obtained This system consists of (1) 0 1 ml of normal human

* This work was supported by grants from the Kress Foundation Lillia Babbitt Hyde Foundation Mary Lasker Fund and Eli Lilly and Company

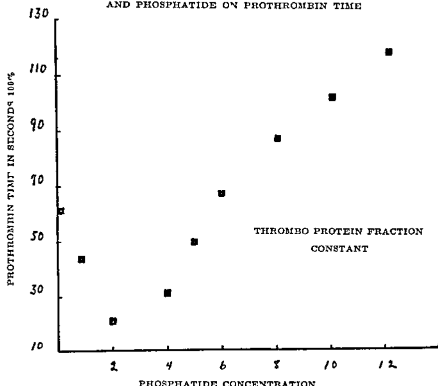


FIG 4

oxalated plasma (2) 0 1 ml of saline (0 85 per cent) containing a constant amount of alpha (obtained from the residue after removing the inhibitor) (3) the designated increasing amounts of the phosphatide inhibitor I As one can see, the addition of 0 1 ml of calcium chloride (0 025 M) to this system, when I is not added results in a prothrombin time of approximately 60 seconds When 0 1 mg of I' is employed in this system the prothrombin time is shortened to approximately 40 seconds in the presence of the same amount of calcium chloride With the addition of 0 2 mg of the phosphatide fraction the prothrombin time is approximately 20 seconds With increasing amounts of the phosphatide fraction the prothrombin time is prolonged as shown in Figure 4 In considering this type of curve one is reminded of the prothrombin time activity curve obtained on varying the calcium ion concentration as shown in Figure 5

EFFECT OF
CALCIUM CONCENTRATION
ON PROTHROMBIN TIME

PROTHROMBIN TIME IN SECONDS 100"

40
30
20
15
10
5

01
02
03

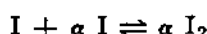
C Cl₂ CONCENTRATION

FIG 5

In explaining the curve in Figure 4 we have postulated an equilibrium between the phosphatide inhibitor I and the thromboprotein 'alpha



$$I + \alpha \rightleftharpoons \alpha I$$



$$I + \alpha I \rightleftharpoons \alpha I_2$$

For instance I (phosphatide inhibitor) plus alpha (thromboprotein) is in equilibrium with α I which is so-called thromboplastin An excess of I will act with α I forming α I_2' which is also inhibitory Since alpha is inactive by itself it evidently forms in the presence of I a protein complex which has thromboplastic activity In order to explain the minimum in Figure 4 I shall consider a hypothetical system in which we have four molecules of alpha The addition of one molecule of I

to four molecules of 'alpha would give first one unit of thrombo plastic activity The addition of two "I's" to four "alphas' would give two units of thromboplastic activity This proceeds according to the following equations

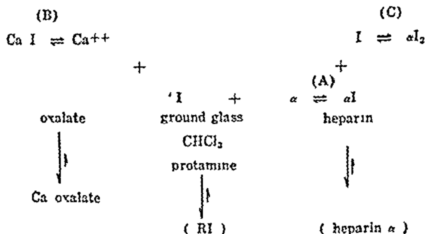
(1) "I" + 4 "α' = 1 unit of thromboplastic activity
(2) 2 I' + 4 α' = 2 units of thrombopl istic activity
(3) 3 'I" + 4 'α" = 3 ' " " "
(4) 4 'I" + 4 "α" = 4 " " " '
(5) 5 I" + 4 "α' = 3 " " "
(6) 6 "I" + 4 "α" = 2 " " ' '
(7) 7 'I" + 4 'α" = 1 unit ' ' '
(8) 8 'I + 4 α" = 0 "

The maximum of thromboplastic activity corresponding to the minimum of the prothrombin time curve occurs when the two substances are present in equivalent amounts (equation 4) This is one possible explanation for interpreting the activation of 'alpha by I and the inhibitory action of "I" depending on its concentration

Since one of the main objects of these conferences is to provoke discussion I thought it would be appropriate to discuss our ideas concerning the mechanism of blood coagulation with regard to this new concept of thromboplastic activity We are primarily con cerned only with the activation of prothrombin and not with the reaction of thrombin on fibrinogen to form fibrin However, the end point of all our experiments is the formation of the fibrin clot so we cannot completely ignore this reaction and as will be pointed out below it must be considered in evaluating some of the data

As previously indicated the equilibrium reaction between 'I' and "alpha to give 'alpha I' may be controlled by adjusting the concentrations of alpha and I as shown in Figure 4 Exactly how this is controlled in vivo is not known at present Theoretically one should be able to control this equilibrium in many ways The most efficient way of obtaining maximum thromboplastic action is by the addition of thromboplastin and calcium ions to the blood or plasma system With the addition of oxalate or heparin one obtains the opposite or anticoagulant action An attempt to explain

these various actions and some of the other questions is outlined below schematically



Equilibrium A is of primary importance because it controls the activation of prothrombin by supplying αI (thromboplastin) Alpha I (thromboplastin) is necessary for the activation of prothrombin B and C are important controlling equilibria which probably function in vivo and which can be influenced experimentally by in vitro experiments

The actual mechanism of the action of the calcium ion is unknown However the classical theory considers its presence to be absolutely necessary for the activation of prothrombin Hammarsten [*Z techr f physiol Chem* 22 *333 (1896)*] first established that the presence of calcium is necessary for the formation of thrombin and that it was not essential for the action of thrombin on fibrinogen Sabbatani [*Arch Ital de Biol* 36 *397 (1900)*] postulated that citrate and oxalate bound the ionizable calcium which they assumed to be necessary for coagulation Nordbe [*Skand Arch f Physiol suppel* 11 *75 (1936)*] was able to remove calcium without adding the usual anticoagulants (oxalate citrate) Coagulation was prevented by this technique however with the addition of calcium coagulation was obtained He also showed that the velocity of coagulation increased with increasing calcium ion concentration up to a certain limit confirming the work of Mellanby [*J Physiol* 38 *28 (1909)*] and others that the coagulation time reaches a minimum at a definite optimum

calcium ion concentration Higher concentration of calcium ion decreased the speed of the coagulation process Scheuring [*Biochem Ztschr* 227 *437 (1935)*] and Ferguson [*Am J Physiol* 123 *341 (1938)*] found that higher calcium concentrations inhibited the formation of thrombin while Weitnauer [*Biochem Ztschr*, 288 *137 (1936)*] was unable to demonstrate any definite inhibition

We do not think that *calcium is necessary for blood coagulation* Its action is involved in controlling the equilibrium (A) as shown above For example if oxalate is added to blood calcium ions are removed from this system This will shift the Ca I'' $\rightleftharpoons$ Ca^{++} + I'' *equilibrium (B) to the right thus liberating molecules* of I' into the blood The high concentration of "I in the blood is actually what is responsible for the so-called anticoagulant action of oxalate ions, since the excess 'I' liberated removes α I'' (thromboplastin) from the system shifting equilibria (A) and (C) to the right with the formation of α I_2'' In Figure 5 the effect of increasing calcium concentration is plotted against prothrombin times The addition of small amounts of calcium ions increases the rate of reaction as measured by the prothrombin time and a minimum is obtained With additional amounts of calcium ions an inhibition or decrease in the rate of reaction is observed The curve in Figure 5 can be divided into three portions The first portion is that dealing with the increase in the reaction velocity with the addition of increasing amounts of calcium Second is the point or portion of maximum activity and third, the portion of inhibition or decreasing reaction velocity with the addition of increasing amounts of calcium The addition of calcium ions to this system shifts the equilibria C A and B to the left At the start the system principally consists of α I_2' The addition of small amounts of calcium corresponding to the first portion of the *curve increases the concentration of* α I This is responsible for the shortening or increased velocity of the prothrombin activation reaction The second portion or point of maximum activity represents the amount of calcium ion required to *adjust the equilibrium A so that a maximum concentration* of α I is present. The third portion of the curve or that portion where inhibition takes place can be explained by the fact that equilibrium B then controls the rate of reaction The increasing amounts *of calcium ion then remove* 'I from the system forming 'CaI This reduces the concentration of I in the system thus

decreasing the amount available for the formation of 'α I Since Figures 4 and 5 are similar, the effect of adding increasing amounts of calcium could be looked upon as similar to the addition of I to the system as in Figure 4 until maximum activity is obtained

The inhibition caused by increasing amounts of ' I or calcium to the system however is due to a different phenomenon (that is equilibria shift in two different directions) As previously shown the addition of increasing amounts of I remove α I from the system forming α I_ thus decreasing the concentration of α I *The addition of increasing amounts of calcium removes* I , thus decreasing its concentration and likewise the concentration of 'α I causing a decrease or inhibition of the reaction velocity

As previously stated it should be possible to adjust these equilibria by controlling the concentration of I and α so that *α I is present in optimum concentration for the activation of* prothrombin As we reported last year at this conference we were able to activate prothrombin in diluted (12 5 per cent) oxalated plasma with crude streptomycin HCl [Overman R S *Macy Foundation Conference on Blood Clotting and Allied Problems* 1 *144 147 (1948)*] This activation was accomplished without the addition of thromboplastin or calcium ions If prothrombin were removed by BaSO4 or inactivated by heat, coagulation will not occur This interesting observation stimulated our interest in the activation of prothrombin by other mechanisms and substantiates our ideas on the action of thromboplastin.

It has been claimed that prothrombin may be activated to thrombin without the addition of calcium and thromboplastin This has been observed with chloroform [Howell *Am J Physiol* 26 *453 (1910)*] ethyl alcohol carbon tetrachloride [Cekada *Am J Physiol* 78 *512 (1926)*] trypsin and certain proteolytic viper venoms [Eagle and Hains, *J Gen. Physiol* 20 *543 (1937)*] The explanations offered by these investigators and others have been varied and the isolated observations fundamentally have not thrown any light on the blood coagulation mechanism

In our chemical work we have found the phosphatide inhibitor soluble in chloroform, carbon tetrachloride and ethyl alcohol containing small amounts of water The phosphatide fraction also has a very strong affinity for glass ground glass Nuchar (kindly supplied by West Virginia Pulp and Paper Co New York City),

fuller's earth asbestos and under certain conditions sucrose (data to be published) This information indicated to us that chloroform and the other organic solvents were removing ' I , the phosphatide fraction from the system This of course shifts equilibrium C to the left and increases the concentration of α I" causing coagu lation to occur If the concentration of these organic solvents is too high, no coagulation takes place because they have then effec tively removed α I ' from the system through their action on "I A curve very similar to the curve in Figure 4 is obtained when in creasing amounts of chloroform are added

We have tried various other means of controlling the concen tration of I" and ' alpha in the blood system Since our experi ence with the clotting of diluted (12 5 per cent) oxalated plasma with crude steptomycin HCl would not take place in whole plasma we resorted to using the dilution technique We found that whole blood diluted to 10 per cent with saline (0 85 per cent) would not clot spontaneously on standing over 24 hours However, on adding ground glass Nuchar fuller s earth or sucrose to this diluted blood system spontaneous clotting occurred These observations can be explained similarly I ' being removed by adsorption The addition of greater amounts of adsorbent then prolongs the clotting time as in the case of calcium ions

The question of why blood clots more rapidly in glass tubes than in silicone or lusteroid tubes might be commented on here briefly The first phase of blood coagulation is the one that is altered since blood added to thrombin in glass silicone or lusteroid tubes causes clotting in the same time We believe that the glass tube acts in much the same way as ground glass does and removes I from the system thus increasing the concentration of 'α I' Silicone or lusteroid has no effect on I It remains in the system and prevents the blood from coagulating as rapidly This difference in the rate of coagulation in these two types of tubes is a good in *dication of the high activity of the phosphatide inhibitor* The *amount* that can adsorb to the surface of a glass tube which is filled with 1 ml of blood is extremely small yet it is capable of prolonging the coagulation time of 6 10 minutes in glass to over 30 minutes in a silicone tube

The mechanism of the activity of trypsin and other proteolytic viper venoms in the blood coagulation process is unknown Because of the similarity between the effect of trypsin and of calcium-thromboplastin on the prothrombin thrombin conversion Eagle

assumed that calcium and thromboplastin together form a pro teolytic enzyme which reacts with the prothrombin We are in clined to think that these proteins are similar in their chemical structure and behavior to alpha The addition of compounds such as trypsin and viper venom increases the concentration of alpha like compounds This increases the concentration of the lipoprotein α I the α' now referring to trypsin or a viper venom component This would explain the normal prothrombin times obtained in the presence of the phosphatide inhibitor using viper venom as the thromboplastic agent [Overman R. S *Macy Foundation Conference on Blood Clotting and Allied Problems* **1** *144 147 (1948)*] Studer [*Volume jubilaire en l honneur de M Emile Christope Barell* Bale *(1946)*] did a very excellent piece of work on just such a system, and he was able to prepare a very active water soluble thromboplastin by combining a lipid fraction with trypsin

The exact mechanisms by which heparin and protamine act have been problems in blood coagulation since the time of their discovery The anticoagulant action of both heparin and protamine and the neutralization of the anticoagulant effect of heparin with protamine can be explained in a very logical manner by applying the above theory

It has been reported that heparin forms protein complexes Examples of this are protamine and various other proteins found in the blood [Best C H & Jaques L B *Ann New York Acad Sci* **49** *501 (1948)*] We do not believe heparin is an anticoagulant itself but that due to its chemical or physical properties it is capable of removing alpha from the blood system This liberates into the system I which is actually the material that maintains the fluidity of the blood the heparin alpha complex itself being inert The addition of protamine to heparinized blood neutralizes the effect of heparin and with approximately equal quantities of protamine and heparin a normal coagulation time is obtained However if an additional amount of protamine is added protamine then acts as an anticoagulant The neutralization of heparin by protamine is the generally accepted explanation for the progres sive shortening of the coagulation time on adding increasing amounts of protamine However the anticoagulant action of addi tional amounts of protamine has not been explained

These facts can be explained by utilizing the equilibria which we believe are responsible for controlling thromboplastic activity

fuller's earth, asbestos and under certain conditions, sucrose (data to be published) This information indicated to us that chloroform and the other organic solvents were removing ' I", the phosphatide fraction from the system This of course shifts equilibrium C to the left and increases the concentration of ' α I ' causing coagulation to occur If the concentration of these organic solvents is too high no coagulation takes place because they have then effectively removed α I' from the system through their action on "I A curve very similar to the curve in Figure 4 is obtained when increasing amounts of chloroform are added

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The mechanism of the activity of trypsin and other proteolytic viper venoms in the blood coagulation process is unknown Because of the similarity between the effect of trypsin and of calcium thromboplastin on the prothrombin thrombin conversion Eagle

large differences in the anticoagulant activity of these two materials Theoretically heparin should be much more active than protamine since the removal of one alpha from the system actually manifests itself in a decrease of two units of thromboplastic activity when alpha and I are present in equivalent amounts in the system (see equation 4) while the removal of one molecule of "I" by protamine only reduces the thromboplastic activity by one unit

This is an attempt to logically explain the factors which are involved in the activation of prothrombin Many of the points discussed here will require years of work before they are definitely established In the interpretations of our findings we have had to discard some concepts that have been considered as fundamental in the field of blood coagulation We have postulated an equilibrium reaction to explain thromboplastic activity This consists of a phosphatide fraction I and a protein fraction α which are in equilibrium with α I (thromboplastin) The activity of the thromboplastin or the rate of activation of prothrombin depends upon the concentration of α I in the system. We do not believe that calcium ion is required for the activation of prothrombin However, we believe that the concentration of α I (thromboplastin) can be controlled by varying the calcium ion concentration Other factors such as heparin and protamine may vary the concentration of the constituents necessary for the activation of prothrombin

DISCUSSION

Wright We ask the members of this Conference not to inhibit themselves in the expression of new concepts I believe the first two speakers have certainly lived up to this credo We are ready for a free discussion of their presentations I saw Dr Seegers busily taking notes along with a number of others Would you like to open this discussion?

Seegers I want to ask Dr Tocantins a question in regard to the transition he made from the work on the brain material to the work on plasma It seemed to me that it involved the assumption that the material was the same in one as in the other How valid is that assumption or am I mistaken in even calling it an assumption?

The addition of protamine to heparinized blood does not inactivate heparin but ties heparin in a protamine heparin complex releasing "alpha" into the system The amount of "alpha" released depends on the amount of protamine added This then combines with I' forming thromboplastin With increasing amounts of protamine increasing amounts of "alpha" are released and the rate of coagulation returns to normal The anticoagulant effect produced by adding more protamine to the system can be explained by the hypothesis that protamine then acts on "I", removing it from this system The decrease then in the concentration of I in the system decreases the amount of "α I" This slows up the rate of reaction of the coagulation process and explains the anti coagulant action of protamine The anticoagulant effect of heparin and protamine on the prothrombin times of diluted plasma (12 5 per cent) is shown in Figure 6 From this it is easy to see the

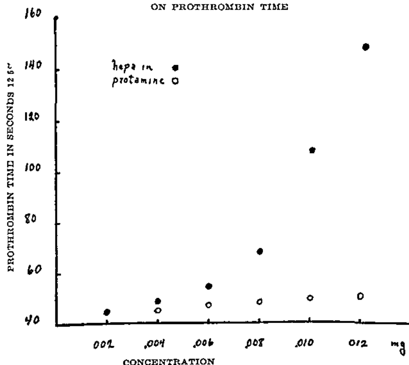


FIG 6

Overman I do not believe that calcium is necessary for the activation of prothrombin It is my idea that there should be many ways to change the concentrations of these two materials in order to obtain an optimum concentration of thromboplastin Since calcium seems to have an affinity for the inhibitor it is an important factor in regulating the equilibrium In other words the ratio of I to a determines the rate of prothrombin activation The different rates in the coagulation times of blood in silicone and glass tubes can be explained by shifts in the concentration of I This inhibitor activator is present in very small amounts in the phosphatide fraction Probably Dr Tocantins can corroborate this It is not a molecule which sticks to a definite behavior in any fractionation procedure and it is hard to evaluate just what type of compound we are dealing with There are many compounds present in the phosphatide fraction which have not been considered before The work of Dr Carter at Illinois and the work at the Regional Laboratory at Peoria have shown that this fraction is extremely complicated

Seegers Did I hear correctly that you got clotting without calcium in oxalated systems

Overman We have been able to activate prothrombin in oxalated systems without the addition of calcium or thromboplastin I reported last year at this conference that diluted (12 5 per cent) oxalated plasma clots in the presence of crude streptomycin HCl (Merck) Coagulation does not occur when whole oxalated plasma is used That brings out the point that Dr Tocantins raised on dilution Dilution or the effect of dilution on other factors present, seems to help to adjust this equilibrium and plays an important role The first compound we obtained spontaneous clotting with was crude streptomycin HCl Since then we have obtained clotting of diluted blood with ground glass carbon etc as mentioned in my presentation

Tocantins I can corroborate that point If to twice citrated plasma kaolin is added it will clot without the addition of calcium There is always the objection that there is some calcium in kaolin Even allowing for that by an excess of citrate it will still do it

Wright Doctor Smith and Doctor Link were asked in advance if they would be prepared to discuss the papers of Doctors Tocantins and Overman We will now hear from them

Tocantins I thought I stressed it—I should have stressed it a little more than I did that the two systems (i e the brain ex tracts and blood) are entirely different, of course Is that what you have in mind—the effect of dilution on the two of them?

Seegers Are you dealing with the same substance in both instances?

Tocantins As far as we can tell the blood and plasma anti thromboplastins have the same solubility characteristics as the brain lipid antithromboplastin Neither gives any of the reactions for protein or carbohydrate Since all these fractions are relatively crude, the melting points have not been tested We don't know anything about those They may turn out to be different Grossly the brain plasma and plasma fraction extracts appear alike

Seegers Actually you don t know? There is an assumption involved?

Tocantins Their physiological behavior is the same Their behavior towards thromboplastin and the other components of the clotting system is the same

Seegers I want to ask Dr Ralph Overman a question as to whether or not the inhibitor is derived from lung thrombo plastin Chargaff s work deals with a lipoprotein, we believe Is your inhibitor derived from that type of lipoprotein?

Overman Chargaff reported that the inhibitor that he isolated was present in the sphingomyelin fraction Is that what you have in mind?

Seegers It is not at all clear to me whether or not you are thinking in terms of thromboplastin as being lipoprotein

Overman Yes

Seegers Do you start with thromboplastin to get your in hibitor?

Overman Yes I start with active thromboplastin which has a prothrombin time of 15 seconds on normal whole plasma and 38 seconds on diluted (12 5 per cent) plasma The phosphatide frac tion is isolated by extraction We remove the lecithin and sphingo myelin fractions and then fractionate the purified cephalin fraction That is where we have found the most active material

Seegers The other question I wanted to ask is whether or not you believe the calcium is necessary for the activation of prothrombin?

proves to be thermostable, like heparin He said that the curves with his material and the heparin differ a little but I think it should be stressed that he is dealing with a complex mixture containing many impurities Can it be that the divergence between the curves is related to the complex nature of the environment and that the material is, after all heparin?

The second paper contains rather imaginative concepts concerning thromboplastin and certain inhibitors The original mixtures unfortunately were complex It is to be hoped that the active fractions will be obtained in pure form so that simplified systems can be prepared with a view toward studying the products of reactions and the reaction mechanics involved

Wright I think we should ask Dr Tocantins and Dr Overman to discuss Dr Smith s comments because we would like to all hear what evidence if any there is that this is not heparin

Tocantins Just as Dr Smith said there is a striking parallel between the method of separating our inhibitor and the efforts of Howell McLean and Holt in the same direction Just as we did they started out with what they thought was an ether extract—a phosphatide Later however they gave up that method of extraction and went to saline extracts of the liver We start with a lipoid solvent instead of saline Furthermore there is very good evidence that our inhibitor is not heparin For one thing protamine will not inactivate it In fact the addition of protamine to a clotting system containing the inhibitor in variable amounts will simply prolong the clotting time as protamine often does in a system where there is no heparin That seems almost to exclude heparin from consideration

The second particular bit of evidence against this anticoagulant s being heparin is its lack of antithrombin activity It would be very difficult in our minds to reconcile that with the known antithrombic activity of heparin. In fact as I pointed out in the chart, the addition of this inhibitor to a system for testing antithrombin activity by the orthodox method simply discloses a diminution in antithrombin activity rather than an increase This has been true with even our most purified preparations The most purified preparations that we have had are as simple anticoagulants of the order of approximately 30 to 50 times less potent than heparin milligram per milligram but even those preparations do not have any antithrombin activity and are not neutralized or inactivated by protamine

Smith In summary the papers of Drs Overman and Tocantins are concerned with the first phase of coagulation a phase in which thrombin is formed We are dealing, furthermore with substances spoken of as coagulants or procoagulants and other substances known as anticoagulants The older work of Morawitz and Howell indicated that one procoagulant was cephalin, or at least one of the lipids Later the protein type of thromboplastin was emphasized particularly by Mills in the late 1920 s Then the two thoughts were combined It was then agreed that we are dealing with lipoprotein

Reference has been made to the work of Chargaff who dealt particularly with this problem There still remains the question as to whether lipoprotein is the sole answer or whether there is both a lipoprotein variety and a lipid material such as cephalin having an independent action In brief, are there two thromboplastins or essentially one? This is a problem which I think cannot be answered at the present moment The discussion this morning reminds me of the early papers from Howell s laboratory The first of these was the paper of J McLean in the American Journal of Physiology 1916 Four years later, in the same journal there was an article by Howell and Holt They found that two fractions could be obtained from the ether extracts of various organs One was a procoagulant and was rich in cephalin The other an alcohol soluble fraction associated with lecithins was apparently an anti coagulant In the process of purification with the aid of alcohol Howell discovered that the previous alcohol soluble fraction be came insoluble What apparently happened was that they were eliminating the lipid material after which another material was precipitated This material becoming insoluble after many pre cipitations was their primitive heparin It was rich in phosphorus and they spoke of it as a lipoprotein inhibitor

As heparin became more carefully purified it became obvious that it was not a lipoprotein The methods by which Howell and Holt and McLean and others had separated out this inhibitor were so similar to the methods which have been used by the speak ers this morning particularly by Dr Tocantins that I have been wondering for some time if his product contained heparin I have been led to think that this material was not heparin because of Dr Tocantins previous report on its thermolability However Dr Tocantins now tells us this was a mistake Instead we are really dealing with a complex mixture which on homogenization

ought to have some idea whether these men have become involved in heparin chemistry What do you think about it Dr Best?

Best This is not the first time that the suggestion has been made that we change the name of what we now call heparin McLean's paper describes something that probably was not the same thing that was eventually purified and crystallized The present evidence does not indicate that what Tocantins is working with now is heparin The matter I feel is in a confused state However we cannot go back over the McLean Howell and Holt matter again The purified material we now use is known the world over as heparin and we should purposely retain the name in spite of the opinions and objections to it When Tocantins material is purified the situation will be clearer

Smith Has the name heparin been copyrighted?

Best I do not think so All the commercial heparin is now made from lung It is not made from the liver One of the first things we did in Toronto was to go over the different source materials and certainly all the large lots of heparin were made from lung The name heparin is thus misleading but is justified by the history of its development

Wright Are there any further comments?

Link This is more in the nature of a practical question on the work presented here by Overman and Tocantins Is there any way of making suggestions at this stage that have a bearing on thromboplastin preparations for the prothrombin determination? Obviously many of these variations that one runs into in practice with thromboplastin preparation are tied up with this question I wonder whether Overman or Tocantins have some specific suggestion to make how to help stabilize that situation in the light of what they know today

Tocantins I have no definite suggestions We have thought about this a great deal We have been impressed by the difference in what we call the activation curves of these different thromboplastins extracted under different conditions There is a great deal of that in the literature Dr Astrup mentioned that an extract of thromboplastin stored for two or three days may give the same clotting time obtained originally but that its behavior on dilution was different suggesting that something had happened to the thromboplastin Perhaps some inhibitor was freed Certainly the balance between the inhibitor and thromboplastin has been altered

When we first found this anticoagulant was heat labile, we immediately excluded heparin from consideration Since we found that the material is fairly heat stable it might seem at first sight to put it under the same category as heparin That is about the only thing we found it has in common with heparin It does not give any positive reactions for carbohydrate It is true that heparin in a combined form with lipid may be masked Even the most purified preparations that we had however, do not give the reaction for the presence of carbohydrates That is as far as the evidence goes Some of this evidence was presented in the original paper on the lipid anticoagulant [*Proc Soc Exp Biol Med* 69 *431 (1948)*]

Overman There is only one comment in addition to Dr Tocantins' We have isolated this inhibitor activator from the phosphatide fraction of soybeans as well as from plasma and brain and lung thromboplastin The material isolated from the phosphatide fraction of soybeans has approximately the same activity as that isolated from brain and lung thromboplastin I do not believe that heparin is present in this particular fraction because here we are dealing with a plant phosphatide that has been thoroughly processed commercially Heparin if present in soy beans, should have been removed in the meal

Tocantins I should like to add one point that the concentrations which were used in testing the antithrombin activity of this material ranged all the way from 0 1 mg per cent to 50 mg If anything as one went up the scale antithrombin activity was reduced something which was a little disturbing to us because we did not want to have an inhibitor which actually enhanced thrombin activity But we are fairly certain that there is something in our preparations which does that separate and distinct from the inhibitor At any rate there is no evidence of antithrombin activity even in concentrations as high as 50 mg in the system

Wright We will now ask Dr Link to open the next phase of the discussion

Link Dr Wright for all practical purposes the discussion of Dr Smith—and I assure you there is no collusion between us—took away the query I had in my mind namely whether or not we are dealing with the heparin preparation or heparin like preparation It seems to me the best thing I can do is to pass the question over to the heparin experts here Dr Best for instance certainly

they like Howell originally are dealing with heparin—I think that the only possible idea which could be advanced in favor of this view is that Howell was originally and perhaps the present workers are dealing with a compound between phospholipid and heparin Such a compound of course may have quite different physical and chemical properties from heparin as we know it and may probably even have different properties with regard to various reactions in the clotting system That however is purely speculation At the moment, in fact we know very little about the properties of compounds of heparin with the phospholipids However it does suggest that the settling of this problem will not be quite as easy as Dr Smith suggested That has not been our experience In fact, it has been our experience that isolated heparin in the form of a sodium or barium salt has quite definite characteristic properties but on the basis of those solubility properties one cannot necessarily predict the solubility properties of heparin in tissue extracts There is also the further difficulty (one we have had in trying to isolate heparin from human blood) we found a difference in the heparin obtained from different species Whatever that means fundamentally we don t yet know Certainly it is of great practical importance in trying to carry out isolations

I have several comments to make with regard to the question of the practical standardization of thromboplastin I would like to draw the attention of the group to the recent results reported by Macfarlane and Biggs where they point out that, with a marked decrease in prothrombin the prothrombin time becomes very sensitive to the concentration of thromboplastin In such a case undiluted thromboplastin gives a prolonged prothrombin time whereas if the thromboplastin is diluted the prothrombin time is much shorter This suggests that the inhibitor present in thromboplastin preparations is of great significance in the standardization of thromboplastin for prothrombin time determinations

I would like to ask Dr Overman how Chargaff s thromboplastin is related to his substance With regard to Dr Overman s observation on clotting in the absence of calcium I would like to mention one experiment It is an observation that MacDonald and I made some years ago in the course of a series of investigations doing prothrombin times for various purposes We routinely tested our thromboplastin on normal plasma Not only did we do the ordinary prothrombin time but also at the same time ran a

At the state of my knowledge I would not have any constructive suggestion as to how to tackle the problem mentioned by Dr Link

Overman I think that the best thing to do at present is not to treat thromboplastin in any way that will disturb the equilibrium ('I + α ⇄ α I'') Dr Quick originally reported that he was unable to *increase thromboplastic activity by any means other than* acetone extraction of rabbit brain tissue This seems very logical for it is my experience that both the phosphatide and the protein fractions, which are the basic components of the thromboplastin molecule, are insoluble in acetone The concentration of the con stituents necessary for thromboplastic activity can be increased by the removal of undesirable impurities with acetone extractions In using any other method of purification thromboplastic activity is decreased because of the removal, due to solubility factors of one or the other of the components necessary for thromboplastic activity Our problem now is to isolate this material in crystalline form Once this is done we can then possibly answer definitely some of the questions that have been raised The effect of the calcium chloride concentrations on prothrombin and coagulation times and the effect of chloroform on oxalated blood have been reported by various laboratories and were first observed many years ago The clotting of blood in silicone and glass tubes also demonstrates the effect of a change in the concentration of this in hibitor Another interesting observation concerning this is the paper by Charles Fisher and Scott [*Trans Roy Soc Can Sec* V, 28, *49 (1934)*] where they found at one stage of the isolation pro cedure in the purification of heparin that they increased the heparin unitage by a considerable amount They investigated the alcohol extract and found a coagulant which they, on further purification, could not justify as being cephalin Fischer and Hecht [*Biochem Z 269 115 (1934)*] made similar observations They isolated a coagulant which was not cephalin So it seems we are dealing with a compound which acts both as a coagulant and as an inhibitor, depending upon its concentration in the system. One can look upon this inhibitor as a coagulant if the concentration is in the proper range This might explain why these investigators have been able to isolate fractions which have coagulant activity and which they do not attribute to cephalin

Jaques I have a number of comments to make and questions to ask First—regarding Dr Smith s suggestion that a possible explanation of Dr Tocantins and Dr Overman s studies is that

a clotting time of 25 minutes is obtained on calcification Let it stand a little longer and the clotting time on calcification will drop on down to 10 minutes and finally to approximately 5 minutes Then the clotting time remains at this particular time If this same experiment is done except that ground glass or chloroform is added to the diluted blood before calcification one obtains the same type of curve but less time is required The clotting time comes down to a minimum but if we add more ground glass or chloroform the clotting time is prolonged and finally no coagulation is obtained on the addition of calcium chloride We feel that all we are doing is removing I and that this can be done just by letting blood stand in a glass tube or by the addition of ground glass or chloroform to this system

In other words you have decreased the I present so that the concentration of alpha I is ideal for maximum coagulation Then if more I is removed say by ground glass or chloroform this decreases the concentration of alpha I causing a prolongation in clotting time If all of the I is removed from the system no coagulation occurs

I think it is very interesting that you have been able to take thromboplastin and add that preparation to oxalated plasma and obtain coagulation because theoretically that is the way it should work As yet we have not found the right conditions for this particular type of experiment

Wright Are there any further comments from anyone?

Tocantins I should like to mention at this point regarding what Dr Overman just said about the effect of ground glass and the glass surface itself on the inhibitor that that has been our experience glass apparently adsorbs or inactivates this inhibitor from plasma If one takes plasma and places it in contact with glass say for two or three days or puts ground glass in it and tries to assay the antithromboplastic activity one finds minimal amounts of it The same things happens with kaolin We have been unable as yet to extract this material from the glass itself or from the kaolin Apparently only very small amounts are present I hate to be giving the impression that we are agreeing on so many things Dr Overman and I I have been looking for some disagreement but so far I have not been able to find it Perhaps when I look into his theoretical explanation a little closer I will find points of disagreement

second sample of thromboplastin with plasma with no added calcium We found that our thromboplastins, when the rabbit brain was first isolated and stored gave a normal prothrombin time with added calcium, (12 seconds) and that this thromboplastin in the absence of calcium did not coagulate the oxalated plasma However after storage of rabbit brain in vacuo in a desiccator for ten or twelve days we found that the thromboplastin prepared from rabbit brain clotted the oxalated plasma in 20 to 30 seconds without the addition of any calcium We thought there might be contamination of calcium We found that storage of thromboplastin over sulfuric acid in the desiccator caused clotting of the plasma more effectively than did thromboplastin stored over calcium chloride It was accidentally observed because of the fact that we were running this extra control I wonder if this would be of any significance or value to Dr Overman

One other point for Dr Overman—is it possible to summarize his viewpoint by saying that what one has is substance "I the actual reactant and a competitive inhibition due to excess of the reactant and to think of it that way rather than plotting it as an inhibitor?

Overman Do you have in mind the relationship between our 'alpha' fraction which we consider thromboprotein and Dr Chargaff s fraction which he has isolated by centrifugation?

Jaques Yes

Overman We have not done any experiments in respect to that We expect to try to use his technique and add his fraction to our phosphatide fraction to see if that acts the same as the material we have isolated from thromboplastin

In regard to the thromboplastin which you prepared and let stand did you let it stand in glass tubes or in silicone tubes?

Jaques This was in glass tubes The experiment was done seven or eight years ago

Overman We find that glass has a great affinity for this phosphatide fraction It adsorbs or inactivates the material in some manner One can dilute whole blood to 10 per cent with physiological saline solution and obtain no coagulation within 24 to 48 hours This bloood is not taken under any special conditions and is kept in glass tubes If calcium chloride is added to this diluted blood, a clotting time is obtained of say arbitrarily 30 minutes If we let the diluted blood stand for 10 minutes then

GENERAL DISCUSSION CONCERNING THE STANDARDIZATION OF THROMBOPLASTIN

Edsall I have been thinking of a question raised by Dr Best at the first conference—*how can one get standardized preparations* of thromboplastin to give a quantitative meaning to the term thromboplastic activity In the case of the thrombin unit there seems to be no serious problem any more The activity of thrombin has been standardized That is the easiest part of the system to standardize Everything else comes to the determination of throm bic activity in the end In order to really determine thrombo- plastic activity I should think one would need a system containing all the components involved in the activation of prothrombin to thrombin preferably in purified form and all of them except the factor that one is specifically studying in optimum amounts to make the reaction go in optimum speeds Until lately we were a very long way from knowing even what the factors were much less purifying them I think Dr Smith has pointed out there are still probably some factors which are only guessed at and not definitely known as existent But it seems to me it is fundamental to the determination of antithromboplastic activity that one should have a way of measuring and calibrating thromboplastic activities What worries me is we don t seem to have that yet in adequate form

Wright It is very interesting and perhaps of major signi ficance that Dr Tocantins and Dr Overman have been able to duplicate their work so closely considering the difficulties which have been presented The question of the standardization of thromboplastin is a very pressing one for both laboratory and clinical purposes We have tried to encourage such a development

Smith I believe Dr Wright you are thinking of a different problem from the one Dr Edsall was discussing Up to this point we have been thinking of thromboplastin from the standpoint of an isolated chemical substance to be used with other isolated puri fied factors It is essentially a problem in pure science I think the other problem you have raised is one of getting something that is suitable for assay work in the clinic There one accepts a compromise and deals with crude mixtures of many things hoping that the right amounts of all factors are present It is something like making clam chowder We get several batches and they are

Flynn I would like to ask Dr Tocantins why he calls his inhibitor an antithromboplastin? It seems to me it may act on some of the other factors particularly since nothing happens when this material is added to thromboplastin Apparently an essentiality for its demonstration is the use of plasma It makes me suspicious that perhaps it reacts with one of the factors concerned in the conversion of prothrombin to thrombin

Tocantins Regarding the actual nature or the mechanism of action of this inhibitor, perhaps we are as you say assuming too much by calling it an antithromboplastin It certainly does not seem to have a direct inactivating effect on the thromboplastin without the presence of plasma Incubation of the inhibitor with thromboplastin in the presence of plasma does reduce the activity of the thromboplastin It may have an effect on other factors such as the platelets Whether as you say it is an antiprothrombin or anti accelerator factor remains open We have experiments in progress now through the kindness of Dr Seegers who supplied us with purified prothrombin and Ac globulin, to try to decide that point There are some things which suggest that perhaps it is an antithromboplastin and not an anti accelerator or antiprothrombin For one thing the progressive antithromboplastic effect is difficult to explain on the basis of its being one of the latter—the fact that if one adds the inhibitor to the plasma and then allows the inhibitor to be in contact with it plus the thromboplastin for 30 minutes before recalcification the clotting time is further delayed It does seem as if the inhibitor ties up with some material in the plasma which enables it to reduce the effectiveness of the thromboplastin and one can show a gradient in the development of that reaction from zero time to 30 minutes The fact that the inhibitor does not block the activation of prothrombin by Russell viper venom seems to be against its being an antiprothrombin

Fremont Smith I am very glad that Dr Best made his comment with regard to insulin because it illustrates the impurity of pure science Apparently we have relativity perhaps all along the line One questions whether one can always make quite as sharp a dichotomy as one wants realizing that even the most pure crystalized substances have impurities in them It seems to me that in a complex situation like the thromboplastin one we ought to work simultaneously towards both the goals Dr Smith stressed

Ferguson The problems raised by Dr Tocantins paper and the comments we have heard so far prompt me to a few general remarks on the thromboplastic mechanism of blood clotting It is gratifying it seems to me that Dr Tocantins is pursuing the topic of the phospholipids which permit of a definite chemical approach whereas nearly all recent work on coagulation has gone back to the use of crude thromboplastin preparations The latter are mere aqueous (or saline) tissue or cell extracts the purification of which is either not attempted or carried only to a crude preliminary and empirical degree of fractionation

Chargaff to whom we have alluded this morning has endeavored to characterize a macromolecular lipoprotein thromboplastic agent from both tissue and plasma sources His analyses give us data which we must accept in part at least but I believe no biochemist or any of us working in the coagulation field can feel with any real conviction that Chargaff s materials are definitely characterized Dr Flynn will have something interesting to say on this later on in these conferences For the last few years in our own laboratories we have used brain and other tissue thromboplastins almost exclusively Our fibrinogen and prothrombin etc are now much more highly purified than hitherto but we have only a few inadequate recent data [*Blood* 3 *1130 (1948)*] obtained with the use of purified cephalin whereas ten years ago when our prothrombins etc were very crude we nevertheless secured highly significant data on the thromboplastic effectiveness of this phosphatide The dependence of thrombin yield as well as of the prothrombin activation rate upon the concentration of the thromboplastic phospholipid was I believe clearly indicated in data published in 1938 [*Am J Physiol* 123 *341 (1938)*] a year or two before the effects of varying thromboplastin (lung extract) concentrations on the thrombin formation from purified prothrombin were reported by Mertz Seegers and Smith [*Proc Soc Exp Biol* and *Med* 42 *604 (1939)*] Much experimental analysis and

more or less alike, but the clam chowder is still clam chowder I believe these are two totally different problems, both very worthy aims in themselves but they ought to be kept separate in thinking

Wright I think everybody recognizes that they are two separate objectives If the attaining of the first objective is some thing for the future, the attaining of the second objective, which is easier may be a practical and important step We have a responsibility, those of us in this field to provide some type of a standard or nearly standard substance which is suitable for practical use If the chemists after 5 or 10 years are able to provide us with a crystalline substance which is more satisfactory so much the better The first can be abandoned in lieu of the second My understanding is that we do not know how long a period it will be before the attainment of the first is a possibility Maybe you are closer to this than I recognize Do you have the idea that it will be a relatively short period of time?

Smith I don t think so

Best We have made two standards in Toronto for the United Nations—one the insulin standard and the other one the heparin standard Having worked with such beautiful crystalline sub stances as these one would probably hesitate to enter into a field where when one purifies the material it get less potent Let us examine this a little more thoroughly Crystalline insulin the world standard has two factors in it one is anti diabetic and the other acts in the opposite direction Happily, there is not much of the factor which acts in the opposite direction It is Berger's glucagon and it can now be removed from insulin The removal does not make as much difference as might have been predicted because the clinician never knew and cannot tell now the difference between the two preparations There may be one or two units difference per mg It is very interesting physiologically to have glucagon available for study

I think the same thing may apply to crystalline heparin I am not sure—none of us is but it does not seem correct physiologically that heparin should cause agglutination of platelets Maybe some day we will find that really pure heparin does not do this So one is after a little thought brought to the viewpoint that it might be useful to have a standard of thromboplastin even though it turns out that the first material has several different things in it I feel that even a crude standard would serve a useful purpose for a time

that so often crop up unexpectedly in any experimental research arousing interest at the time and then so often escaping into the limbo of the past because of lack of opportunity for following them through

To return to the more specific problems raised by Dr Tocan tins report I should like to recall the occasions when during the use of brain cephalin as a thromboplastic agent we frequently noticed that too high a concentration always became inhibitory [*Am. J Physiol* 123 *341 (1938)*] We had a good deal of experience with the stability of cephalin preparations Kept under absolute alcohol they remained usable for several years, but did show a gradual loss of activity despite retention of the creamy whiteness that indicated absence of (brownish) oxidation The thromboplastic activity could never be standardized owing to variability of the preparation This variability was particularly observed when different techniques of preparation were compared The biochemical criteria of *thromboplastic* phospholipid have never been satisfactorily established Fischer and Hecht [*Biochem Ztschr* 269 *115 (1934)*] Charles Fisher and Scott [*Trans Roy Soc Can Sect V* 28 *49 (1934)*] and Chargaff [*J Biol Chem* 155 *387 (1944)*] all obtained data particularly with reference to alcohol solubility and perhaps resistance to oxidation which raise some doubts as to whether the thromboplastic phospholipid can be identified with cephalin It is true however, that ordinary cephalin as prepared from brain by the classical extraction procedures of lipid chemistry and analyzing close to P N (NH_2) and C ratios of a typical phosphatidyl ethanolamine (i e cephalin) was demonstrably thromboplastic in concentrations of less than 1 1 000 000 in our early work *(op cit)* In 1943 we made similar tests on four lipid fractions obtained from brain cephalin by Dr Jordi Folch at the Rockefeller Institute, N Y These data which have not previously been published are appended in Table 9 Folch [*J Biol Chem* 146 *35 (1942)*] gives the following data concerning fractions the percentage referring to yield in gm per 100 gm original cephalin mixture V = phosphatidyl ethanolamine (15%) III = phosphatidyl serine (27%) I = inositol phosphatide (22%) IV = unidentified mixture (8%) In personal communications he stated that III and I could contain about 5 10% of V (true cephalin) and IV may very well turn out to be either identical with or closely related to phosphatidyl ethanolamine Thus the biochemical criteria were

thoughful interpretation went into our efforts to formulate a simple explanation of the activation of prothrombin to thrombin as proceeding via a protein + phospholipid + calcium *intermediary* complex [*Am J Physiol*, 123 *341 (1938)*] The chemical possibilities of reactions between a protein and a phospholipid and a metal (calcium) were obvious The evidence for the participation of calcium in an 'intermediary was rather striking at that time [*Am J Physiol* 119 *755 (1937)*] and much of this evidence has recently been confirmed *in experiments on highly purified prothrombin* [*Blood* 3 *1130 (1948)*] That the final thrombin retains activity in preparations containing no detectible calcium (Hammarsten Ferguson) or P lipid (Howell Astrup) is a fact of general acceptance We adduced evidence in our early work [*J Lab Clin Med* 26 *52 (1940)*] that the ubiquitous phospholipids indiscriminately tied up with the various proteins present were often not "available' for participation in the reaction of thrombin formation It was clearly evident from all these studies that an additional factor or factors in thrombin formation had to be sought Our particular direction of enquiry into this led us along the path to the plasma proteolytic enzymes and this had proved an extremely thorny path beset with many difficulties and uncertainties I do not therefore wish to go into these problems at this session but would like to suggest that we retain an open mind about the possibilities of proteolytic *enzymes existing in many of our test systems and contributing* in some measure to certain of the results obtained [*Blood* 3 *1130 (1948)*] More recently [*Proc Soc Exp Biol and Med* 67 *228 (1948)* *J Clin Invest* 27 *778 (1948)*] we have been glad to follow for a way the strikingly new path opened up by the work of Owren Ware Seegers et al Quick Fantl and Nance etc which points clearly to the participation in the thrombin forming mechanism of a globulin type protein (see later) From the start of our enzyme work we have kept in touch with the hemophilia problem particularly with reference to the 'thromboplastic action of pancreatic trypsin (experimentally) in thrombin formation in hemophilic plasma [*Am J Physiol* 126 *669 (1939)*] and the ability to demonstrate protease on suitable activation of the ordinary crude (human antihemophilic globulin [*Proc Soc Exp Biol* and *Med* 64 *285 (1947)*] We are still working on the enzyme problem in its *many aspects* and also attempting to follow the lines of divergence encountered in the studies cited We mention these things largely to point out the numerous and curious observations

The (weak) thromboplastic activity of Folch s fraction I is interesting in view of Dr Overman s suggestion that his inhibitor probably contains inositol Obviously little significance can be attached to inositol when one type of preparation containing it is active and another preparation inhibitory It may also be recalled that de Süto Nagy [*J Biol Chem* 156 *433 (1944) Am J Physiol* 141 *338 (1944)*] found anticoagulant (antithromboplastic and antithrombic) phosphatides in the albumin fraction of tissue extracts He identified them with the sphingomyelin group of phosphatides with the realization that it (the term sphingomyelin) does not imply a chemical entity and that not all sphingomyelins are anticoagulants His final preparations did not contain sulfur [*cf* Chargaff *J Biol Chem* 121 *175 (1937)* 125 *677 (1938)*] and he raised the possibility whether admixture of sulfur-containing cerebrosides described recently by Blix [*Z physiol Chem* 219 *82 (1933)*] might have accompanied the anticoagulant sphingomyelins obtained by Chargaff *e g* from nervous tissues These remarks will serve at least to indicate the difficulties and complexity of the phospholipid problems in coagulation chemistry

Returning for a moment to crude thromboplastins I should like to mention that we have noted on a number of occasions that if too great an effort is made to get a maximal amount of material (acetone-extracted brain tissue) into suspension a less potent thromboplastin may result Our present routine is to place 200 mg of dried brain thromboplastin into 5 cc of borate buffer solution [*Blood* 3 *1130 (1948)*] and stir only occasionally in a water bath at 37 C for ten minutes We often discard this first extract and use the second fraction prepared similarly by renewing the buffer solvent In one recent test where the first extract was obtained with too vigorous stirring it gave a 19½ *sec* clotting time (prothrombin time) with Dr Quick s test on a certain plasma whereas a similar batch less well suspended gave a 15 *sec* PCT test We could only conclude that the difference must indicate some antithromboplastic inhibitor which could be increased by certain methods of extraction With the tremendous significance of thromboplastin both to clinical applications and to investigative work on blood coagulation it is deplorable that we must use as a key reagent something as fickle and undefined as thromboplastin I have no answer to this problem except to renew the pursuit of the phospholipid factors in the coagulation reactions Dr

not fully adequate for a definite correlation with the thromboplastic activity qualitatively demonstrated by our very sensitive test, which as shown in the table, detected such activity in all four fractions, although none of them was as potent as a weak buffered suspension of cat brain thromboplastin Moreover all the preparations supplied were dried and brownish from months of exposure to the atmosphere and must have lost a considerable amount of their thromboplastic potency Inconclusive as they are these tests nevertheless add some evidence to indicate that true cephalin is a potent thromboplastic phospholipid Any other conclusion will need further advance in the field of phospholipid chemistry

TABLE 9

THROMBIN FORMATION IN PRESENCE OF VARIOUS THROMBOPLASTIC AGENTS FROM BRAIN TISSUE

Thrombic mixtures (T) = 4 cc prothrombin + 0 5 cc borate buffer + 0 25 cc N/10 CaCl + 0 25 cc cited agents incubated for periods indicated *Clotting times* (sec)* at 25 C pH = 7 75 for 0 5 cc T + 1 0 cc prothrombin free fibrinogen The P lipids are 1 1000 suspensions in borate buffer

T	Thromboplastic Agent	Incubation Time (min at 25 C pH = 7 75)						
		10	20	30	60	90	120	150
0	(Calcium only)	±	±	±	±	+	+	+
1	Brain Susp	45	28	27	27	27	28″	30
2	P lipid V	+	320′	180′	68	53″	48	49
3	P lipid III	+	760	340	108	78	76	78″
4	P lipid I	+	570′	380″	140′	90″	82	82″
5	P lipid IV	245″	120″	70	38	39″	40	42

* + = clot starting in ¼ –1 hr and becoming solid later
± = weak clot starting in several hours and incomplete in 24 hours

Ferguson Yes it is in this section that I think we may profitably return to details of what wetting adsorption, and other surface phenomena may mean to the complex plasma system in which not only coagulation, but also changes in residual formed elements (especially platelets) and in the plasma proteolytic enzyme mechanisms are all influenced by factors of this kind

Conley I think it is very important to recognize the significance of this inhibitor in thromboplastin preparations as far as the determination of prothrombin time itself is concerned If I may, I would like to emphasize that simply by a graph on the board [from *Am J Med Sc* 215 *158 (1948)*] illustrating some results we obtained a couple of years ago when we were trying to find out what does cause thromboplastins to vary in their activity We determined the prothrombin clotting time of a normal plasma varying the concentration of the rabbit brain thromboplastin that we were using With increasing concentrations of thromboplastin there was definite inhibition of clotting which presumably was associated with the presence of the inhibitor in the thromboplastin preparation

We were interested then to study in the same way plasmas from dicumarolized patients i e plasma with reduced prothrombin concentration. When we did that we got a parallel curve at a higher level When the prothrombin concentration of a normal plasma was reduced by diluting it with prothrombin free plasma prepared by barium sulfate adsorption the resultant plasmas gave a curve which could be superimposed on the dicumarolized plasma curve However if we took a normal plasma and diluted it with saline we got a very different curve The inhibitor in the thromboplastin appeared to be much more effective in the plasma diluted with saline than it was in the plasma diluted to the same extent with prothrombin free plasma This suggests that there must be something in plasma itself which antagonizes the inhibitor This is confusing but very important because many people use saline dilution curves in the determination of prothrombin activity When one is dealing with a plasma with a low prothrombin concentration there is a big difference between the calculated prothrombin concentration expressed as per cent of normal depending on whether a reference curve based on saline dilution or prothrombin free plasma dilution is employed The differences between saline and prothrombin free plasma dilution curves are in part attributable to the difference in concentration of fibrinogen and Ac globulin

Tocantins' work is pointing in this direction and is, I believe a real contribution from this standpoint

On the matter of Dr Tocantins' reliance on collodion coated and lusteroid (relatively "non wettable') tubes to support the hypothesis of an "antithromboplastic' plasma factor said to be weakened by dilution, I have repeatedly raised the question whether the data might not be equally well explained not by the removal of an inhibitor but by the provision of more favorable conditions for *activator* effects that are not optimal (e g in collodion tubes) even in the normal plasma and still less so in the hemophilic My point is that the evidence for any inhibitor should be unequivocal and I, personally prefer to stay with the idea of a deficiency of activator (or of conditions favorable to activation) until this simpler explanation is clearly proved inadequate to account for the experimental facts

Wright Perhaps Dr Tocantins would like to comment

Tocantins It isn t clear to me just what is meant by the question I grant that conditions were certainly more favorable for coagulation The very fact that the rate of coagulation was accelerated is evidence of that But it is difficult for me to think that that favorable state induced by dilution was due to the addition of a coagulant because salt solution certainly does not have that and if anything the known coagulants fibrinogen, Ac globulin prothrombin *and even platelets were all reduced by dilution* So presumably if the conditions are more favorable to coagulation one could not explain them as due to any activation of any known coagulants The other alternative is to explain it as a diminution in the effectiveness of an anticoagulant and since anticoagulants can be reduced in effectiveness very greatly by dilution I think that the explanation lies in that direction

Wright Do you have another question Dr Ferguson?

Ferguson I have just this clarifying comment—that I stress the qualitative if not quantitative, similarity of the phenomena in wettable and non wettable tubes Where I take issue with Dr Tocantins is in the belief that wettable surfaces offer specially favorable situations for thromboplastic activation and that the evidence for postulating any plasma inhibitory factor on the basis of the facts before us seems to me highly equivocal I think the topic may be allowed to rest at this point

Wright We have another section of the conference on surface effects

satisfactory cause confusion Clarification comes only by specification of the exact situations

Wright Would you continue Dr Brambel and follow Dr Fremont Smith s suggestion?

Brambel I will set up the specifications that we follow First we have to be free of hemorrhagic complications Second we have to assure the clinician that there will be no thromboembolic complications at the prothrombin levels to be maintained We believe that the one-stage method as we use it meets these conditions and I think the clinicians here will agree that these specifications are indeed rigid I am very much interested in changing over to a two-stage method if it can be put on a production scale but thus far I have not seen a two-stage procedure that can be used on 175 to 200 bloods a day

Smith The question of mass production reminds me of an analogous situation which arose years ago with the Wassermann serological test for syphilis First it seemed impossible to do the test on a mass production scale But it was done by routinizing the procedure with standardized equipment and reagents Those of us who use the two-stage method have made no real effort to set it up on a quantity basis for 175 determinations a day Many times we have had technicians doing 30 or 40 determinations a day but this cannot be considered mass production I think the number of procedures could be doubled or trebled provided one has standard reagents standard equipment standard reactions I dare say that 175 determinations could be done by two technicians but it will take someone probably six months to work out mass production techniques Be that as it may the one stage method does have some merit True it is a rough and ready test but often very useful I have collaborated for years with men interested in the practical side of hematology Usually their requirements are not too exacting Some clinical men however wish to carry out at least a portion of their work on a more analytical basis In such cases the two-stage determination will be of value but it is a mistake to demand all clinicians to use a two-stage method

The one-stage method will be discussed a little later by Dr Flynn We will see some of its shortcomings a little more clearly but even with its shortcomings I will stress again that it has some merit We have used the one stage test for many years

Brambel Are the shortcomings of the one stage method far greater than the shortcomings of the two stage method?

But there is also an important effect brought about by the increased response of saline diluted plasma to the inhibitor contained in thromboplastin

Seegers I should like to remark that I believe an accurate assay for thromboplastin can be made with the use of purified prothrombin Ac globulin fibrinogen and calcium.

The two-stage method for prothrombin analysis is getting to the point in perfection where I believe it is in some respects easier than the one stage method and I feel it is time for clinicians to take this into account and consider the possibility of making more general use of this method

Brambel I would like to know why you think the two stage method is better than the one stage method for mass production in the clinic I would like to know how many tests you could do at a maximum in a day with your two stage method

Seegers I think you can do more than you would anticipate You can do them with smaller quantities of plasma and with less waste The two stage prothrombin assay lets you know where you stand After all why should a patient go to a hospital with the attitude that it is a dangerous place to go He ought to go there with an idea that the people there know what they are talking about

Brambel The answer to that I feel is the one stage method and it brings up as far as I am concerned a very practical problem of the number of tests that can be done per person That is why I am raising the question We have had very adequate results with the one stage method as we have developed it Complications have been at a minimum

Smith How many do you have to do a day?

Brambel We do as many as 175 a day How many technicians would it take Dr Seegers to run 175 two-stage tests a day

Fremont Smith Let me interject a comment These differences of opinion are due to using the word adequate without precise specification The word adequate must be qualified—adequate with respect to what? The same applies to satisfactory —satisfactory with respect to what? A procedure may be perfectly adequate for some given situation but not for another situation When one investigator has a given situation in mind and another investigator a totally different situation such words as adequate and

We have deviated a little bit from our original discussion I don t think we can close without giving Dr Quick a chance to make a few comments He has been very restrained

Quick As I listened to this discussion I could not help wondering whether some of us may not still be in the 19th Century of blood coagulation in view of the fact that we continue to assume that the reaction between prothrombin and thromboplastin is the first step in coagulation I am fully convinced that this reaction is not the first stage and that there is no free thromboplastin in the blood but that it is present in an inactive form which is activated by the platelets I shall discuss this in much greater detail when I come to my particular subject. We must be very careful when we talk about thromboplastin and prothrombin because many things have happened in the blood before thromboplastin and prothrombin react and therefore one has to be cautious in interpreting results obtained in glass in contrast to collodion or silicone coated vessels because we know the one clotting element which is affected by surface is the platelet I am not sure that any other component is affected very much by surface I recognize that this is a moot question but one has to bear in mind that we do know that the platelet is very definitely influenced by surface and that we also know that it is very difficult to completely remove all the platelets When we do as Dr Seegers will agree we get a blood or a plasma which is practically incoagulable I have postulated that platelets contain a material which activates the thromboplastinogen of the plasma Therefore we have to question when we hear a discussion such as given by Dr Tocantins whether his antithromboplastins are true antithromboplastins or whether they are inhibitors of the activator of the thromboplastinogen There is a condition a hemophilia like disease, in which I have definite evidence that there is a substance which antagonizes the platelet enzyme This again I shall probably discuss at greater length when I present the subject of the function of the platelets

It also has to be pointed out that we have to be very careful in using the term purified reagent Very often one has to ask the question is it a purified reagent or a denatured reagent? When very drastic methods are used in the isolation and purification of materials are we getting substances which are the ones that actually participate in the coagulation mechanism?

There is also another point to consider and it is that we place too much confidence in the coagulation time especially when applied

Smith Unquestionably!

Flynn It seems to me there are two problems under discussion Dr Brambel made the point that the one stage method is a good test for a specific type of problem, namely anticoagulant therapy The experience of many clinicians has shown that the one stage method does afford an excellent control for anticoagulant therapy Dr Seegers made a plea to supplement the use of the one stage test with the two stage technique thereby obtaining more precise analytical information I do not believe Dr Seegers wishes to replace entirely the use of the one stage method Isn t this precisely the thing that Dr Fremont-Smith just pointed out? The one stage method is effective for some things not effective for other things

Wright Most of the anticoagulant therapy in this country utilizes the one-stage technique

That does not mean that it is perfect or that it is the ultimate but it does mean that it can be used safely and satisfactorily If we carry this to the other extreme what the clinician is looking for is a test simpler than the one stage method in the laboratory and it is not beyond the realms of possibility that such may be achieved In fact we have been working with a modification of Dr Smith s bedside test which has made it possible for our fellows to control the care of patients with dicumarol satisfactorily without using the Quick method in the laboratory However, before we release this information, we are going to ask other institutions to use it in order to determine whether they can do the same We are too cautious to release it just now From the clinician s viewpoint the next step in the application of dicumarol on a widespread basis rests with a simpler test not on a more complicated one However there are many cases in which one wishes to know much more than the one stage test reveals about the patient s blood and especially how his blood reacts to anticoagulant therapy Perhaps it is suspected that that patient has liver disease or kidney disease or that there is an hereditary, a familial shall we say discrepancy or unusual balance in prothrombin activity so that several members of the family have prothrombin times either faster or slower than average For such patients we need the information which can be obtained by all methods This is where the two-stage method seems to the clinician to have its greatest application —in the puzzling cases the difficult ones The average or routine cases can be treated by using the simpler methods

I was very much interested in the work of Dr Overman in his attempts to purify thromboplastin Back in 1932 and 1933 I began my studies on trying to make a thromboplastin of uniform potency and I did what he has done more recently—I determined the effect of various solvents Whenever I used a solvent in which cephalin was soluble I obtained a thromboplastin of lowered potency When I used acetone much to my surprise the potency of the thromboplastin increased and that enabled me to prepare a thromboplastin which was stable and very uniform in potency You may call it a crude extract but remember that this thromboplastin prepared from rabbit brain by the standardized method which I have developed has given me results that are amazing in their constancy Since December 1937 when I first began preparing this material until today I have had no trouble what soever I still have thromboplastin which I put into evacuated vials in February 1938 and if any one of you will come to visit me in my laboratory I shall open one and demonstrate that it still has the potency that it had when I put it in It may be interesting to relate that one of the students from Europe who came to work with me was dubious about the constancy of this thromboplastin as some of you perhaps are and I said We shall open this vial which I made in 1938 and test it on the bloods of 25 medical students and see what results we get We took the blood from the medical students and out of 25 24 had a prothrombin time of 11½ to 12 seconds and one had a prothrombin time of 13 seconds I repeated that on several series and occasionally I found an individual with a prothrombin time of 13 seconds So it is possible to find a prothrombin time of 13 seconds in a normal subject but in general it is 11½ to 12 seconds

A word about the crude thromboplastin I might say that if I had been commercially minded and had patented the material as they do in some institutions I probably would be able to supply a thromboplastin which is uniform in potency throughout the United States What happened was this the minute the commercial houses saw how great a demand there was for thromboplastin they went in for preparing this material and none of them sought me as a consultant I don t know why because after all I had prepared the first potent thromboplastin for the use of the one stage method After they had their material on the market many came to me and wanted me to approve their product I have no obligation to indorse any commercial preparation although there is one that

to hemophilic blood This can best be demonstrated by a simple diagram plotting the coagulation time against concentration of thromboplastin At zero concentration the coagulation time is infinity As the thromboplastin is increased the coagulation time rapidly falls and a typical hyperbolic curve is obtained which on the x axis attains a minimum value of 12 seconds irrespective of the excess of thromboplastin It becomes obvious that a very minute quantity of thromboplastin in the region of the steep section of the curve will strikingly reduce the coagulation time In fact I have demonstrated that 0 1 cc of a 1 to 1000 dilution of rabbit brain thromboplastin added to 1 cc of hemophilic blood can reduce the coagulation from 2 hours to 8 minutes Such a small quantity of thromboplastin in the range where the curve is flat would escape detection It is clear that a minute liberation of thromboplastin such as can occur from the injury of clumsily inserting a needle in a vein may be enough to produce a normal coagulation time in hemophilic blood

The logical way of assaying thromboplastin is to add a mea sured quantity to a fixed volume of hemophilic or platelet-free plasma and to determine by the prothrombin consumption test how much prothrombin remains There you have an assay method which really tells you something and is not as indefinite as the coagulation time which has misled all of us since the time of Alexander Schmidt That does not mean that the coagulation time may not be a very good guide for certain purposes but it can be very misleading You know very well that before the prothrombin time was known the coagulation time in cholemic bleeding was reported to be normal time and time again The surgeon operated and the patient died from bleeding simply because they were de pending on this very inaccurate and unreliable coagulation time When the prothrombin time test was developed the whole question was clarified So I think from the point of view of hemophilia you have to be very careful in your evaluation of changes of the coagulation time from two hours to eight minutes That is why progress in coagulation did not occur until we changed from studies in which clotting was determined in minutes and hours to types of experiments in which it was measured in seconds

I think in studies such as Dr Tocantins plasma which is free of platelets as well as from platelet products should be em ployed This means that platelets must be removed before they disintegrate because the products of disintegration cannot be re moved by centrifugation

If those places which I have mentioned can get consistent results why are we struggling so hard to settle on a standardized material?

Another word about the one-stage method It is usually mentioned with some apology as if it were less accurate than the two-stage method One can repeat a thing so often that it finally assumes the *prerogative* of a fact. Let me enumerate what the one stage method has done but, before I do that I want to put this question to all of you Can you name one condition in which hemorrhage occurs due to a deficiency in any part of the prothrombin complex which is not indicated by the one-stage method?

Seegers I think you ought to define what you mean by prothrombin complex.

Quick The prothrombin complex is a group of factors which, with thromboplastin are essential for the formation of thrombin A deficiency of any one of these factors causes an increase in the prothrombin time Any hemorrhagic condition in which the prothrombin time is prolonged can be looked upon as being due to a lack of one of the factors of prothrombin I have yet to find any bleeding condition in which the prothrombin is low as measured by the two-stage method that does not also have a prolonged prothrombin time Let me reiterate what was done with the one-stage method The one-stage method was the first method to disclose the low prothrombin in obstructive jaundice. It also showed the normal prothrombin concentration in hemophilics The one-stage method showed the deficiency of prothrombin in vitamin K avitaminosis a finding that agreed with Schonheyder s conclusion reached with a different procedure The one-stage method very conclusively showed the drop in prothrombin after the feeding of toxic sweet clover hay Both of those investigations were carried out in the summer of 1936 The one-stage method disclosed that there was a new factor that was responsible for prothrombin activation namely the labile factor That work was done in 1943 long before even a suspicion of this factor was suggested by the two-stage method The one-stage method has also disclosed that there are two distinct congenital hypoprothrombinemias which I have studied repeatedly and which work I shall present in great detail when completed I cannot see how the two-stage method can be of much assistance in studying the differentiation between the congenital hypoprothrombinemias

if properly used is fairly uniform in potency All of the rest do not give 12 seconds which I think a standard thromboplastin should give for human blood

I have investigated various brains and I find that the common laboratory animals other than the rabbit, yield a very poor thromboplastin I don't know why The dog the horse the cow are all very unsatisfactory Occasionally the cat gives a fairly good thromboplastin but curiously enough the rabbit and the human brain give a consistently potent thromboplastin The human brain, when carefully extracted with acetone gives a thromboplastin which is actually more potent than that of the rabbit I do not recommend it because the minute we switch from rabbit brain to human brain we have to revise much of our data and also the interpretation of our data because human brain is more active I see no reason whatsoever why rabbit brain cannot be accepted as the standard material

Let me quote some experiences that I have had I went to Tacoma two years ago in May, and Dr Larsen who is the pathologist at Tacoma General Hospital asked me to show them how to prepare a thromboplastin for the prothrombin time test I went to work with only their material which included a Washington rabbit and prepared the thromboplastin, they brought a normal blood to be tested I got a prothrombin time of 11 seconds It happens that a very fresh thromboplastin often gives a prothrombin time a little shorter than 12 seconds but it quickly stablizes to the 12 second potency When Dr Larsen visited me this June he said Quick since you have been out in the Northwest we have had no trouble whatsoever Not only that but we have taught most of the laboratories in the Northwest to prepare thromboplastin and to do your prothrombin time and they are all getting what you claim'

At the University of Michigan I notice they are now using my one stage method as I originally described it and they are getting 11½ to 12 seconds Dr Gilbert Professor of Medicine at Northwestern University, visited me about two or three years ago and brought along his technician to whom I demonstrated my test Soon after that I met them at a Central Society meeting They rushed up to me and said, 'Ever since we have been down to your place we are getting 11½ to 12 seconds They told me recently that they are beginning to do some missionary work around the Chicago area to get others to do the prothrombin method as I described it

to say, We will try our best to obtain results similar to this so that work done in other laboratories can be compared with the minimum of difference of figures'

Tocantins I would like to say something in regard to the remarks Dr Quick made concerning our presentation To begin with the allusion to the presentation as being back in the 19th Century is a little misleading because some of the concepts that we are now working with were actually enunciated in the 19th Century or close thereto It is only since a great deal of stress has been put on prothrombin that the so-called classical or Morawitz theory of blood coagulation involving only two phases the conversion of prothrombin to thrombin and fibrinogen to fibrin has been generally adopted Before that such workers on blood coagulation as Dr Howell and Dr Bordet recognized the existence of three phases in blood coagulation the first phase having to do with the maintenance of the fluidity of blood Bordet thought that prothrombin was in an indifferent state (proserozyme) in the blood perhaps in combination with an inhibitor and it was only when the inhibitor was stripped off that the prothrombin acquired a different state and could then be changed into thrombin We are all familiar with Dr Howell s theory which had three phases the first being concerned with the neutralization of anti prothrombin So many of the ideas that we are now adopting again are simply a reproduction of ideas held by the older workers We are actually making progress by going backwards in other words

As to the one-stage method being the first to demonstrate that prothrombin in hemophilia is normal in quantity I beg to differ because Addis showed in about 1911 that the fundamental defect in hemophilia did not have to do with a quantitative diminution in prothrombin and Dr Eagle in 1933 and 1935 showed not by using the one stage method but by quantitative thrombin deter minations that there was a difference in the *rate* of conversion of prothrombin to thrombin He stressed the fact that the amount of prothrombin was normal and did so without the aid of the one-stage method The one stage method simply came along to demonstrate something in hemophilia which was generally accepted as true

Quick I think Dr Tocantins misunderstood me That was a parenthetical remark I said that the method was the first to determine a prothrombin deficiency in cholemic bleeding and I

As far as the bedside method is concerned I might remark that I used the technique of that test in 1936 in an article published in the American Journal of Medical Science I have not approved of the bedside method because the coagulation time of an opaque medium such as whole blood cannot be done accurately It takes at least a few seconds to detect a clot If the prothrombin time is worth anything it is worth doing accurately and getting prothrombin times that can be checked within half a second I talk feelingly on this subject because the one stage method has been belittled ever since its introduction as being a method which is not exact much of my work stands or falls on the one-stage method and I feel confident that I shall continue to be able to defend adequately the one stage method as we gain more knowledge

I am curious why, when the question of standardization of thromboplastin was brought up that I was not consulted After all the one stage method as it is run in most laboratories is either the method which I developed or some modification of it and I have done I believe considerable work on thromboplastin and should know a little about it

Wright Dr Quick when the question of prothromboplastin standardization was considered at our first business meeting you were here

Quick That is the last I heard of it

Wright That is the last most of us have heard about it Nothing has been done

Quick I thought you had gotten in touch with the U S Public Health Service

Wright Dr Best merely contacted them to find whether they could be stimulated to form a committee to study the problem So far we have heard nothing from them For clarification let me add Dr Quick that the present discussion does not stem from dissatisfaction with any particular method The dissatisfaction arises from the situation whereby manufacturers producing thromboplastin do not have an official standard Personally I would not have any objection if Dr Quick s method and his thromboplastin were accepted as a standard providing the results can be consistently reproduced as he says they can It is the reproducibility we want The important thing is that a standard with a certain unitage should be accepted by some official body *pro tem* so that various manufacturers as well as university laboratories would be able

on anticoagulant therapy is a more important consideration I wonder if human brain might be a source of commercial thromboplastin

Quick May I make one more remark? It is in regard to acetone's increasing the potency of thromboplastin That applies particularly to human and rabbit brains which are very sensitive to acetone purification The potency as measured by the prothrombin time in very definitely increased when rabbit brain is treated with acetone Strangely enough chicken brain thromboplastin is not very much influenced by acetone extraction An extract of fresh brain is about as active as the acetone-dehydrated product The effect of acetone extraction varies in regard to the brains of other animals Apparently acetone either removes something of an inhibitory action or in some way potentiates the thromboplastin I agree with Dr Olwin that human brain is a good source of thromboplastin but it has objections which have been mentioned I don't know what the general reaction would be

Flynn Dr Smith made the point that standardization of thromboplastin is somewhat like standardizing soup If one is going to standardize thromboplastin it ought to be standardized with regard to some one property What property should be used? Activity might be selected If the greatest activity is wanted one should use a viper venom lecithin thromboplastin mixture As far as I know this is the most potent thromboplastin existing but would it be useful?

Quick In regard to Russell viper venom and lecithin it has been observed that with dicumarol blood it fails to show the decrease in prothrombin which is indicated by rabbit or human thromboplastin hence it would be very dangerous to use the Russell viper venom as a standard

Knisely It just occurred to me that fibrinogen is species specific Is thromboplastin species specific?

Quick There is a certain degree of species specificity It is always difficult to know whether it resides in the prothrombin complex or in the thromboplastin Chicken thromboplastin is very ineffective on mammalian blood and vice versa rabbit thromboplastin is only weakly active on chicken blood There is obviously a tremendous difference between the avian and the mammalian

Brinkhous In regard to Dr Quick's reference to the use of viper venom lecithin combination in detecting hypoprothrombinemia I wonder if Dr Quick has additional data on this subject

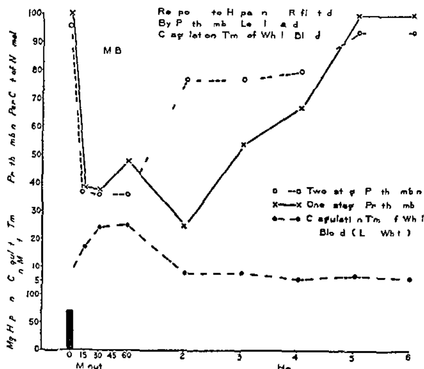


FIG 7

I would like to make just one remark about the standardization of thromboplastin We have been able to repeat Dr Quick's curves and timing very accurately using his method of preparation of rabbit brain We have practically no variation from his normal, day after day, rabbit after rabbit will give 11 to 12 seconds for normal plasma It seems to me that the field of study of the human brain as a source of thromboplastin has not been fully explored We have found that saline extract of human brain frozen at –35 will give an 11 second clotting time over a period of months We have not studied a lot of human brains to know whether or not all of them will do that but the ones that we have studied have shown a fairly uniform reaction While there may be certain objections to that on the basis of offending the sensibilities of some as Dr Quick has mentioned at times in the literature it seems to me that the possibility of establishing a good standard and safe standard in the control of prothrombin levels with patients

the two coagulants acted alike but that when he used prothrombin free plasma as the diluting agent he provided some antagonist which was effective against the rabbit brain thromboplastin but not effective against the Russell viper venom This is our own interpretation of these results

Link From a commercial standpoint there is an element of unreality in what Quick has said Today the most widely used commercial source of thromboplastin does not come from rabbit brain but from the Maltine preparation made from rabbit lung A person may doubt whether the 11½ 12 second prothrombin time in the human with rabbit brain thromboplastin has the ultimate significance that Quick attaches to it for the simple reason that the aforementioned preparation of rabbit lung has a reading of 15 or 20 seconds We ought not to put a figure of 12 seconds into the record here as though it were a meter bar put away in some glass case as a guide for posterity As far as I can see those who use the Maltine material are just as successful as those who use rabbit brain

Quick I might mention that the Squibb preparation which apparently is manufactured from rabbit lung is much more active At least the sample which I had tested gave 10 seconds so if we are considering potency the Squibb preparation is more potent than the Maltine preparation It is a question of getting not the most potent preparation but rather one which stays the same day in and day out

Wright I agree I don t think it is important whether the standard is ten seconds or fifteen seconds or what the number of seconds is but the key question is whether this can be reproduced year in and year out

Best If I were interested in this question purely from the research viewpoint I would try to separate and isolate the various factors I think that is the most fundamental thing that can be done On the other hand I do not think this other interest is incompatible with the primary one at all and I believe that a step forward could be made if someone preferably a government agency took the lead and provided a stable standard thromboplastin which could be thrown overboard the moment anything better was available I think all of us would like to have a government agency accept the responsibility of selecting a group to set up a standard

S J Wilson [*Proc Soc Exp Biol and Med*, *66*, *126 (1947)*] reported a patient treated with dicumarol who died of hemorrhage although the prothrombin level as indicated by the one stage test using Russell viper venom was not greatly depressed It was my impression that if venom plus lecithin is used, the results in the one-stage tests are more nearly comparable to those obtained with tissue thromboplastins

Quick Some of you will recall that at the Buffalo meeting of the International Hematological Society, Macfarlane discussed viper venom I am not certain but I am inclined to believe Macfarlane tested the venom alone and with lecithin S J Wilson is not the only one who made the observation that viper venom is an unsatisfactory thromboplastin for following the effect of dicumarol therapy Cheney in California reported a similar case and Macfarlane stressed this latter case in his presentation at Buffalo

Wright Prandoni and I had a similar experience when we used the Russell viper venom technique We had much more difficulty and unexplained bleeding This was early in the use of dicumarol There has been far less trouble since we have used the other forms of thromboplastin

Tocantins The difference in behavior between Russell viper venom and brain thromboplastin may be due to the fact that Russell viper venom apparently is not susceptible to the antithromboplastin of the plasma You can take the tissue or blood antithromboplastin that we talked about this morning and run it up to concentrations of 50 mg in 0 1 ml of recalcified plasma and if Russell viper venom is added the system will clot just as if there were no antithromboplastin there at all Dr Macfarlane at Buffalo showed that if he took plasma and diluted the plasma with salt solution, he could have curves of activity on adding Russell viper venom which were almost identical with those of adding rabbit brain thromboplastin—that is if he diluted the plasma in both instances with normal saline But if he diluted it with plasma the prothrombin of which had been removed by aluminum hydroxide or other adsorbing agents then there was quite a difference between the two The Russell viper venom clotted the diluted plasma much more rapidly, and the curves varied a great deal from those of the diluted plasma clotted with the rabbit brain thromboplastin This apparently shows that when he diluted the plasma with saline he removed something from the plasma and

11 seconds and 15 seconds If everybody had checked their own cases by normal controls it seems to me they must be close enough to compare their work with Dr Wright's work or the Mayo Clinic work

Wright Dr Smith this problem is a little bit different *Within this group you can see a marked difference in viewpoints* This is why we are here If we prepare thromboplastin from the rabbit brain by Quick's technique we can duplicate his findings fairly well in our laboratory and probably Dr Barker can but if you visit various hospitals that today are attempting to utilize anticoagulant therapy many of them without too much experience and without facilities to prepare their individual thromboplastin and who buy it on the commercial market from different companies you find that their base line figures vary quite considerably

Smith What you need Dr Wright is a law forcing everyone to buy Maltine thromboplastin Apparently some individuals are just too perverse to use the same brand

Wright It is not necessary to have a law It was not necessary to have a law on the unitage of insulin It was only necessary to have a representative organization say that this is our unit and soon everybody used the same unit

Smith If you and Dr Barker want to compare results why don't you use each other's methods?

Wright There are 50 methods or more involved—in other words potentially a different one in each hospital

Jaques I would like to say Dr Wright that the whole question of standardization of thromboplastin is critical at this moment in terms of the development of anticoagulant therapy not in terms of studying hemorrhagic diathesis

For even if you arrange cooperation between the different hospitals laboratories you raise the question whether anticoagulant therapy of the future will be another specialty along with hematology radiology etc or is it going to be part of general medical practice A certain number of my clinical colleagues believe that anticoagulant therapy should be something the general practitioner could use In that case elaborate laboratory tests are out of the question It will be necessary for the general practitioner to have say a thromboplastin preparation in a bottle to which the blood is added Such a bottle would contain the statement that the normal prothrombin time is such and such for therapeutic levels

We should remember that standards, even if they are replaced by better ones in a year or two have served very useful purposes

Smith May I suggest that the standard for minor fluctuations be provided by running a control patient or two now and then? If there are little variations of a second or two, one can make the proper correction That in itself irons out the minor fluctuations that occur in any laboratory

Barker This problem involves more than reproducibility of results in an individual laboratory or institution As a matter of fact there are now many laboratories and institutions in this country where a thromboplastin from one specific source is used consistently and prepared consistently in a certain way by experienced physicians and technicians They recognize quickly any variations in potency of their material and quickly correct them so that the results which they report are comparable and dependable However physicians from different institutions get into trouble when they try to discuss the effects of dicumarol and the optimal degrees of deficiency of prothrombin activity which should be produced by dicumarol The trouble arises because the different laboratories are using thromboplastins of different potency and, therefore the results of the test in terms of seconds are not comparable We thought that we could transpose results in seconds to percentages of prothrombin activity by interpolation on dilution curves and then talk in terms of percentages but we know now that the figures in percentages so determined by different laboratories are not certainly comparable They do offer a better basis for comparison than the times in seconds We have a real problem in communication

Wright That is the way I feel too It isn t a matter of using just patients as controls within your own institution

Barker In almost all institutions where much dicumarol is being used and many prothrombin times are being done each day, normal individuals are tested each day as controls Also dilution curves are checked frequently Also new supplies of thromboplastin are checked against the supply in use by duplicate tests not only on normal plasmas but on plasmas of patients receiving dicumarol who have varying degrees of decreased prothrombin activity

Smith There is no tremendous fluctuation from using thromboplastin prepared by Quick s technique Everybody gets between

procedure to be followed in such standardization it would be ex tremely useful I wonder whether the Cardiovascular Institute or the Heart Institute of the Public Health Service might consider the problem of thromboplastin standardization It might be brought to the attention of the Institute chiefs informally to see whether it is the sort of thing they would explore Perhaps some pattern could be set up to deal with both the chemical and technical prob lems Some idea of the difficulties involved can be obtained by considering that with many techniques—for instance the question of diagnosis of cancer by vaginal smear it is practically impossible for technicians to learn the methods unless the technician is apprenticed to someone already properly trained

I would like to raise another question that comes from one of Dr Olwin s remarks It is perhaps an indication of my ignor ance He said the first sign of bleeding was frequently hemorrhage from the kidneys Can anyone enlighten me as to what is the mechanism by which blood that fails to clot properly should leak from the kidney capillaries

Olwin I think Dr Link might be in a better position to answer the question Work done in the University of Wisconsin showed that the first sign of dicumarol poisoning appeared in the small vessels of the kidney such kidney damage was observed in experimental animals receiving dicumarol Do you recall that work? I think it was done in the Department of Medicine

Link It goes back to the observation which you made originally Dr Jaques with Dr Dale on the dilation of capillaries by dicumarol The work of McCarter followed your work on experi mental thrombosis Very high levels of dicumarol were given to dogs with the deliberate attempt to kill them.

Jaques Both the Wisconsin group and ourselves observed a marked dilation of the veins It was very striking The abdominal and thoracic veins were very markedly dilated

Barker I would like to point out that the hematuria which is encountered occasionally in humans who have received dicumarol may result from bleeding from anywhere in the urinary tract and not necessarily from the glomeruli Many supposedly normal individuals who have not received dicumarol have microscopic hematuria at various times and no lesion can be found in the urinary tract The so-called essential hematuria a more severe type of bleeding is considered by many to come from breaks in

the prothrombin time is such and such for hemorrhagic levels, the prothrombin time is such and such This, I think, is quite a different standardization from what has been discussed up to this point It is something to be kept in mind

Quick I would like to make one more remark There is one factor that we have not considered and that is the training of our technicians We are very much concerned about the accuracy of prothrombin time but other determinations are done just as poorly It should be mentioned that Dr Reed sent an expert in metabolism around a radius of 200 miles of Chicago to have a basal metabolism done on himself His normal was 39 calories per square meter I don t know in how many hospitals he had his basal metabolism performed, but it ranged all the way from 29 to 59, and there were only a few hospitals which determined it accurately

Wright No one can deny that many biological tests have a wide range of error In some diseases certain clinical biological tests are largely of academic interest It still is not a matter of great importance to know that a patient with cirrhosis of the liver has a prothrombin time of Y or Z On our ward rounds we note the fact and pass to the next patient However a different situation prevails with the use of dicumarol therapy Here a fatal result may follow if the prothrombin time has not been determined with a carefully prepared and standardized thrombo plastin Patients do not die if the error in basal metabolism tests is 10 per cent or even 50 per cent but a comparable error may be fatal in dicumarol therapy Furthermore with dicumarol therapy, a prothrombin time error in either direction is important —a false elevation may mean cessation of dicumarol therapy with ensuing thromboembolic complications a false lowering may mean too much dicumarol therapy

Fremont Smith Dr Quick raised the question of training technicians for the prothrombin examination Indeed this applies to a great variety of clinical laboratory tests If the tremendous effort to extend medical care to a large number of the population is successful there will result an increase in the use of clinical laboratory methods throughout the country Concomitant with such an event will arise the question of how laboratory technicians are to be trained and how the various methods are to be standard ized If we can contribute even some small clue to the proper

out—we often find hemorrhage in the hind legs because they jump and traumatize the big muscles in the thighs In newborn pups suffering from severe hypoprothrombinemia I found that they invariably died bleeding into their abdominal cavity

Wright That can be further commented on by the fact that it is relatively common to find the initial evidences of bleeding in the lower extremities proximal to the ankle in ambulatory patients who are on dicumarol One can often see petechial hemorrhages in that area as soon as a few extra red cells appear in the urine or even before

Fremont Smith Is this primarily related to hydrostatic pressure?

Wright That is always a factor particularly in the lower extremities Something additional happens when a patient is under dicumarol Perhaps Dr Smith has given the explanation for the development of hundreds of very tiny petechial spots The reason this finding is not more commonly reported is I am certain because it is not looked for But now that we have 40 or more patients who have been on ambulatory dicumarol for several years we watch for and not infrequently encounter it

Jaques I would like to ask Dr Wright if you have had any examination of the eye grounds because I imagine you would see it there

Wright We have but it has not been very common We have seen a few

Brambel In regard to the eye you see it in the conjunctiva before the eye grounds

There is another comment I would like to make at this time There are a number of cases on record in which the blood was rendered completely incoagulable with dicumarol for some reason with no sign of any external hemorrhage at all Locally I have had a number of these cases called to my attention

Wright That is true in fact it occurred on an idiopathic basis without dicumarol this last week at Cornell University where we had a patient with extreme hypoproteinemia

Fremont Smith Over some period of days or weeks?

Wright This patient had prothrombin times of as long as nine minutes with no prothrombin present according to the two-stage technique We checked the one stage and confirmed that

small veins in the renal pelvis or prostatic area These veins are very close to the lining surface "Normal microscopic hematuria or essential hematuria might be greatly augmented if the patient had prothrombin deficiency but need not necessarily be started by the prothrombin deficiency Hematuria may be the first evidence of bleeding in a patient who has received too much dicumarol, but there are certainly many such patients where the first evidence of bleeding is from some other source indeed some patients bleed severely from some other source without having any detectable hematuria

I believe that either Dr Link or Dr Quick or both of them have stated that the bleeding that occurs when too much dicumarol is given is similar to the bleeding that occurs in hemophilia In both cases there is a serious coagulation defect Such bleeding differs from that which occurs in the purpuric states where the primary trouble is a vascular defect which is usually quite wide spread The bleeding in hemophilias is relatively localized In ordinary daily life we are all subject to minimal injuries in various parts of our bodies including the gastrointestinal tract urinary tract, skin and nasal passages Such injuries may bleed slightly without our being aware of the bleeding But if we have a serious coagulation defect due to hemophilia or too much dicumarol this very slight bleeding may continue until it becomes a major hemorrhage

Wright It should be pointed out that there is bleeding continually in the urine and that Addis counts show that large numbers of red cells are being lost continually Dicumarol therapy may act to increase the number of red cells lost without serious hemorrhage—or massive bleeding may result

Smith While we are speaking of blood coagulation and of vascular injuries in dicumarol conditions we might point out—and I think it is true—that the platelet agglutination is also inhibited by high doses of dicumarol *It may very well be that we* are approaching something functionally like thrombocytopenic purpura, with platelets still present but not functioning in the way they should to agglutinate and stop up small bleeding points from the capillaries That may be more important than the other

Quick There is another factor, age and species Most of the bleeding we see due to dicumarol is in older people whereas in animals for instance in rabbits—I think Dr Link will bear me

Wright You would not want to commit yourself on that?

Brambel No more than to say some cases respond and others don t

Wright Are you sure they responded or were fluctuating coincidentally with the study?

Brambel Some cases that responded were taken off the Vitamin P preparation and there was a return of hemorrhagic manifestations when we attempted to keep up the advanced dicumarol administration

with the two-stage This patient had a negative Rumpel Leede phenomenon as determined by the Wright-Lilienfeld modification This fact may explain the lack of hemorrhage in the face of the absence of prothrombin activity

Brambel I know of a patient who received 1000 milligrams of dicumarol for two weeks and had a prothrombin time of an hour with no sign of bleeding

Fremont Smith You are now bringing out a factor of safe ty which is perhaps saving the lives of many more patients than we realize

Brambel That introduces another problem with regard to bleeding, which may occur at levels which are unpredictable at least in our experience, and I think in yours Dr Wright One frequently finds hemorrhage manifested at prothrombin levels which are apparently safe This is very disconcerting

Fremont Smith There has been some question of the use of Vitamin C along with dicumarol, with the implications that the latter effected vessel fragility Szent Gyorgyi claims to have isolated Vitamin P which he believes was a factor controlling permeability As far as I know it is still a moot question whether Vitamin P is existent or not Has its use ever been explored in dicumarol therapy?

Wright Does anyone here have experience regarding the combined use of Vitamins C and P with dicumarol therapy? I wonder if anyone here has conducted studies along this line

Flynn Isn t there a reference to Vitamin C by Dr Link?

Link Dr Wright has reference to humans I take it Experimentally in at least three species of animals we were able to demonstrate the role of Vitamin C to our satisfaction

Wright I was referring to humans

Link I will say that personally I would not allow any clinician to give me dicumarol until I have the chance to take Vita min C even at Mayo s That is where my faith comes in—faith in the results with experimental animals that Vitamin C plays a definite role (see 1st transactions)

Brambel With regard to Vitamin P we have carried on some half serious studies Some patients have responded and some haven t That is probably due to the limitations of the preparations which are available

according to observations in the electron microscope [C v Z Hawn and K R Porter, *J Exper Med* 86 *286 (1947)* and unpublished work by P R Morrison and by J D Ferry and S Shulman] The strands range in length up to 100 000 A or more and vary in width from 100 A or less to 2000 A whether they are ever as narrow as the diameter of a single fibrinogen molecule is still open to question

As evidence for the hypothesis of end to-end linkage with formation of bonds catalyzed by thrombin we have emphasized the extreme dilution at which fibrin clots can be formed—at fibrinogen concentrations as low as 0 03 g /liter—showing that under these conditions the network strands must be very long and thin An alternative hypothesis which can account equally well for the existence of a network at high dilution is that the fibrinogen rodlets instead of combining end to-end associate side-by side with only partial overlapping (Fig 8 b) The ideas advanced here are developed for the case of Fig 8 a but can also be applied with slight modifications to that of Fig 8 b

The formation of strands in this way would require a re markable precision of orientation The individual fibrinogen molecules are rotating owing to Brownian motion at a rate which we know from Dr Edsall s studies of double refraction of flow corresponds to turning over within a period of the order of 10^{-5} second Such a rod whirling at random about its midpoint would sweep out a spherical region bounded by an area of 1 5 x 10^6 A^2 If the ends of two rods must meet with a precision of 1 A^2 (reasonable for chemical reaction between two specific groups) the chances of correct orientation are less than one in a million Another consequence of the Brownian motion is interference of the fibrinogen molecules with each other The spherical volume swept out by each rod is about two hundred times the volume of the rod itself so above a concentration of 0 5% serious hindrance to rotation should appear and even at the physiological concentra tion of 0 25% (in human plasma) some interference would be expected

Thus the correct orientation both for end to-end junction and for parallel alignment is opposed by thermal motion and steric hindrance But the probability of correct orientation could be increased by the operation of long range forces The only forces

CONSIDERATION OF THE STRUCTURE AND MECHANISM OF FORMATION OF THE FIBRIN CLOT

JOHN D FERRY

Department of Chemistry University of Wisconsin

The problems of greatest current interest concerning the structure and mechanism of formation of fibrin can be expressed I think by two questions (1) What is the geometrical arrangement of the rod like fibrinogen molecules as they fit into the fibrin network? (2) What forces bring the fibrinogen molecules together and what are the chemical bonds if any introduced by thrombin?

There is not much information to add this year to the excellent discussion of these and other problems presented here a year ago by Dr Edsall However, on the basis of known facts regarding the fibrinogen molecule, some deductions can be drawn and some speculations made which may stimulate discussion and experiments

The working hypothesis which has been described before [J D Ferry and P R Morrison *J Am Chem Soc*, 69 *388 (1947)*] is that the fibrinogen rodlets unite end to-end to form strands which are associated laterally in bundles to varying degrees (Fig 8 a) This is consistent with the known dimensions of the fibrinogen molecules which are about 700 Å long and 35 Å in diameter[1], and also with the dimensions of the fibrin strands

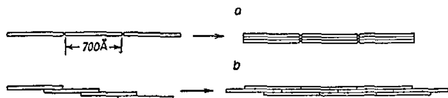


Figure 8 — Possible linkages between fibrinogen molecules in the formation of fibrin strands (a) end to-end bonds with lateral association (b) side by side bonds

[1] These dimensions for a cylindrical rod are probably indistinguishable experimentally from the dimensions of 700 Å x 38 Å calculated on the basis of an elongated ellipsoid [J T Edsall J F Foster and H Scheinberg *J Am Chem Soc* 69 *2731 (1947)*]

employed phosphotungstic acid it is natural to suppose that they correspond to dense concentrations of positively charged basic groups In the arrangement shown in Fig 9 b positive (as well as negative) charges are concentrated at intervals of 230 Å along the fibril

Of course the Coulomb forces could not fix the end to-end associations they would only increase greatly the probability of suitable orientation The permanent bond must be provided by thrombin Since thrombin clearly functions as an enzyme (*i e* it does not enter stoichiometrically into the reaction) we may suppose *that it activates a group on one fibrinogen end (Fig 10 a) Thrombin* carries a net negative charge within the pH range where clotting occurs so it is likely that this group is near a positive *area on the fibrinogen molecule as shown* The activation must last long enough for the thrombin to diffuse out of the way (Fig 10 b) since its bulk (diameter about 60 Å if spherical) would interfere with the final reaction (Fig 10 c)

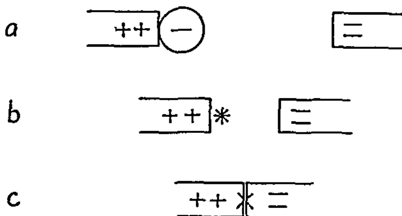


Figure 10 — Proposed sequence of events in the action of thrombin (a) reaction of thrombin with a positively charged region on fibrinogen (b) activated group remaining after thrombin diffuses away (c) junction of oppositely charged fibrinogen ends

This picture can be used to interpret a number of different observations In the first place there are certain conditions which slow down the rate of conversion of fibrinogen to fibrin as measured gravimetrically [J D Ferry J T Edsall P R Morrison

which could conceivably act over the distances involved are electro static

In the pH range within which clotting occurs (from 5 8 to 10) the fibrinogen carries a net negative charge so that at great distances the Coulomb forces are repulsive However the net charge perhaps of the order of 100 electrons per molecule* at pH 7, actually represents a relatively small imbalance between a large number of positive and negative charges, of the order of 500 each per molecule[2] It is conceivable that these charges could be concentrated in such a way that very large forces of attraction would dominate at close range In particular dense groups of positive and negative charges at opposite ends of the molecule would lead to end to-end orientation (Fig 9 a) while bands of positive and negative charges grouped at intervals would cause side-by side attraction (Fig 9 b)

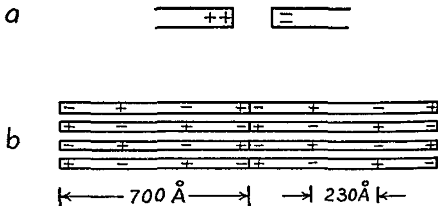


Figure 9 — Proposed scheme of operation of electrostatic forces in aiding alignment of fibrinogen molecules (a) end to-end junction (b) side by side association

The bands in Fig 9 b have been drawn with a spacing of one-third the length of the molecule because this offers an attractive if highly speculative interpretation of the striations of fibrin observed in the electron microscope (C v Z Hawn and K R Porter, *loc cit*) These striations spaced about 230 A apart may represent regions which combine heavily with the electron stain

*These estimates are based on unpublished titration data of J D Ferry and P R Morrison on human fibrin

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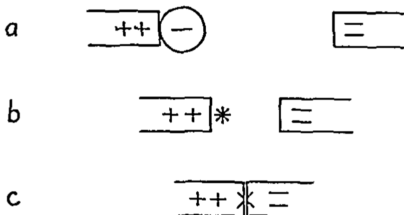


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V Kimel, and W F Lever *Federation Proc*, 6 *250 (1947)*] or as roughly gauged by the clotting time (a) increasing concentration of neutral salt from ionic strength 0 15 to 0 6 (J D Ferry and P R Morrison, *loc cit*), (b) presence of certain anions, such as thiocyanate in concentrations of the order of 0 1 M (J T Edsall and W F Lever unpublished work) (c) increasing concentration of fibrinogen above 0 1% (d) presence of certain neutral molecules such as hexamethylene glycol, pinacol and cyclohexanol (J D Ferry and S Shulman, unpublished work) Speaking generally any circumstances which interfere with alignment and approach of the fibrinogen molecules would be expected to slow down the reaction The detailed interpretations are as follows (a) Neutral salt diminishes all Coulomb forces between protein molecules by the shielding effect of ionic atmospheres and in particular it diminishes the postulated attractions which lead to proper orientation (b) Large anions are often adsorbed by proteins [I M Klotz and other, *J Am Chem Soc*, 70 *2935 (1948)*] in the present case adsorption of thiocyanate by fibrinogen would increase the net negative charge and hence the long range electrostatic repulsion (c) The effect of increasing fibrinogen concentration probably represents steric interference of the rods which increasingly impede each others' rotation as discussed above (d) The neutral alcohols which prolong the clotting time by several fold at a concentration of 1% can hardly affect any long range forces but may be adsorbed in such a way that the specific groups of fibrinogen are prevented sterically from reacting with thrombin or with each other

All the conditions outlined in the preceding paragraph not only delay clotting but also modify the clot structure, yielding finer strands Thus in each case the side-by side alignments are impeded more than the end to end alignments This also seems consistent with the interpretations given although I shall not take time to give the arguments in detail

There is another category of reagents which delay the conversion of fibrinogen to fibrin but produce clots of coarser structure hydrogen ion and guanidinium ion Combination of these substances with fibrinogen would decrease the net charge and the long range repulsive forces and therefore favor alignment in agreement with the experimentally observed changes in structure Their effect on the rate of reaction must be attributed to some thing more specific than the geometrical relationships discussed

above for example combination with some group on either fibrinogen or thrombin which is essential to the reaction between the two It is suggestive that the only protein side chain which combines with hydrogen ion in the pH range where clotting occurs is the imidazole group of histidine

Further information concerning both the forces which bring fibrinogen molecules into alignment and the bonds which fix them there may be forthcoming from studies of substances which inhibit the reaction at moderate concentrations By inhibition I mean prolongation of the clotting time to at least twenty four hours, in practice this is usually equivalent to preventing clotting entirely The following compounds have been found to prevent clotting at concentrations of the order of 5% hexamethylene glycol pentamethylene glycol bis(2 hydroxyethyl) sulfide urea cysteine and ascorbic acid (S Shulman and J D Ferry unpublished work) The inhibition of the coagulation of whole blood by cysteine was reported by J H Mueller and S Sturgis [*Science* 75 *140 (1932)*] The action of these inhibitors is currently under investigation

DISCUSSION

Edsall I have no extensive comments to make about Dr Ferry s presentation mainly because I have been familiar with his work for so long My discussion in last year s conference dealt largely with his studies and I think that Dr Ferry and I are in pretty close agreement on most of the questions which have been raised

I like his picture of the electrostatic mechanism for the side-by side aggregation I still have no more suggestions than before as to the nature of the particular chemical groups that may be involved in the conversion of fibrinogen to fibrin by the action of thrombin Last year I think I said something about the possibility that amino groups may be essential for this process That possibility is still open but, if anything I feel somewhat more doubtful about it than I did then

I might make a few remarks about some of the anions the effects of which on the fibrin clot have been studied by Dr Lever and myself Iodides and thiocyanates have similar effects although the effects of thiocyanates are considerably more marked at any given molar concentration Both iodides and thiocyanates change the character of the clot and make it more translucent and

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carries no net charge we cannot offer the same explanation for these effects as for the others All this of course is just a fragmentary glimpse of a few factors affecting this complex problem

Ferguson I should like to make several comments on this problem of the thrombin fibrinogen reaction First I should like to call the attention of the group to the work of W F H M Mommaerts [*J Gen Physiol* 29 *103 113 (1946)*] Mommaerts pointed out that fibrinogen being a linear (highly asymmetric filamentous molecule) colloidal SOL, can change to a fibrillar GEL not only by end to-end molecular elongation but also by a mesh like lateral association determined by suitable aggregating forces Phenomena involving forces of this type were called coacervation by Bungenberg de Jong (1936) A *coacervate* is a condensed system of colloids between which certain attractive (and repulsive) forces act Electrostatic forces depending upon the surface (of colloid particle) distribution of + and — charges are especially important for coacervation The marked effects of many salts on colloidal reactions is largely explained by the properties possessed by salt ions with respect to altering surface charges on colloidal particles The double valence rule expresses the fact that both the cations and anions of the dissociated salts modify the respective (— and +) charges on the coacervating colloids From the known facts about the electrolytic dissociation of salt their valence ionic radii polarizing power etc the differing effects of various salts on colloidal reactions involving electrical charge can be fairly accurately predicted Applying these ideas experimentally to various salt effects on clotting* Mommaerts concludes the clotting of fibrinogen with thrombin is at least partly caused by a coacervation process due to electrostatic attraction between positive and negative groups

K L Laki and W F H M Mommaerts [*Nature* 156 *664 (1946)*] point out that fibrin clot is not formed when fibrinogen and thrombin are mixed at pH = 5 1 i e below the isoelectric point (pH = 5 3) of fibrinogen Clotting will occur promptly, however when the pH is restored to neutral or slight alkalinity Moreover the longer the mixture is kept at pH = 5 1 the quicker is the

*Some excellent studies of salt effects on the blood clotting mechanisms especially the *inhibitory* types were made a few years ago by Glazko [*ref* A J Glazko Studies on the mechanism of blood coagulation Ph D Thesis Univ of California (19 9) A. J Glazko and D M Greenberg *Am J Physiol* 128 *399 (1940)* A J Glazko and J H Ferguson *Am J Physiol* 134 *54 (1941)*]

more friable than it would be if formed in sodium chloride at the same pH and ionic strength The effect of these anions is more or less equivalent to that of increasing the pH of the solution in which clotting takes place The anion of the salt of acetyl tryptophane has a very powerful effect in the same direction, too Incidentally, Dr Ferry [J D Ferry and R S Gordon Jr, *Federation Proc* 5 *136 (1946)*] has also found that this anion has a very marked effect in inhibiting the gelation of gelatin so that *gelatin can remain fluid in its presence at a much lower tempera*ture that it would otherwise Thiocyanate has a similar effect on gelatin, but somewhat less than that of acetyl tryptophane

It is interesting in this connection that thiocyanates and iodides are well known as denaturing agents for many proteins However their effect on denaturation is generally apparent at only much higher concentrations than those which we have used to produce modification of the structure of fibrin clots Workers who studied the denaturation of proteins generally used at least molar solutions of these salts whereas we have found very marked effects on the character of the clot at concentrations near 0 1 molar

Another well known denaturing agent is guanidine hydrochloride Like iodide and thiocyanate it has a profound effect according to Lever s work in prolonging the clotting time and delaying fibrin formation This effect is markedly apparent even at concentrations as low as 0 05 molar and at 0 1 molar the effect is profound However in another respect its action is opposite to that of thiocyanates or iodides for the clots formed in the presence of guanidine hydrochloride are more opaque than would be expected in sodium chloride at the same pH and ionic strength Qualitatively speaking we can explain the results on the ground that in solutions of iodides and thiocyanates the fibrinogen has a tendency to bind the anions thus acquiring a greater negative net charge and shifting the electrostatic conditions to what they would be like at a more alkaline pH In guanidine hydrocholride on the other hand it is probable that the cation (the guanidinium ion) is bound to the protein more strongly than the chloride ion thus shifting the charge on the fibrinogen to a more positive value, as if the solution had been adjusted to a lower pH

It should be added that another well known denaturing agent urea acts on the fibrin clot like the iodides and thiocyanates at least in a qualitative sense The clots become more friable and translucent in the presence of urea (0 1—0 5 M) Since urea

clotting tests All the test mixtures had the same concentration in terms of *original* salt fibrinogen thrombin and borate buffer (pH = 7 7) and represent a tenfold dilution with distilled water of these agents Clotting times after the cited periods of incubation with 4 5 per cent NaCl are recorded in seconds at 24 C

TABLE 10

"PROFIBRIN" FORMATION IN NaCl FIBRINOGEN THROMBIN MIXTURE

	MIXTURE (with SALT)	ADDED (with water)	INCUBATION PERIOD				
			½ min	5 min.	10 min.	15 min	20 min
1*	Thr + fibrinogen	(water only)	150	85	15	5	
2	Thrombin	Fibrinogen	190	200	205	215	212
3	Fibrinogen	Thrombin	240	245	250	300	295

Mixture (1) clotted in 18¾ *min.*

Note a The phenomenon of Apitz is strikingly evident in (1)

b The thrombin is not significantly affected (2)

c The fibrinogen, however does seem to suffer some alteration in "reactivity as the result of treatment with the salt In all our experiments of this type however the *reactivity* becomes less as judged from the longer clotting times in series (3) as compared with series (2) Quite the reverse is the case when fibrinogen is partly denatured by mild heating

In the second type of experiment typical results of which are summarized in Fig 11 we followed Apitz s technique of heating fibrinogen in 5 per cent NaCl for 12 minutes at 48 C

Clotting times (at 37 C) and fibrin yields (1 hr after the thrombin addition) measured by recovery of the clot digestion with Mehl s biuret reagent and photoelectric colorimetry (standardized against gravimetric and macroKjeldahl data) were tested in parallel experiments at four dilutions (4 3 2 1) of (a) the *profibrin* (heated solution) and (b) a *control* consisting of the fibrinogen plus salt but unheated

clotting on subsequent neutralization These data afford another example of the *two stage* formation of fibrin especially studied by K Apitz [*Z ges exp Med*, 101 *552 (1937)*, 102 *202 (1937)* 103, *417 (1938)* 105 *89 (1939)*] in proposing the idea of a (?denatured) intermediate "profibrin" Fairly strong neutral salts (e g 5 per cent NaCl) do not prevent the initial thrombin fibrinogen inter action (? *profibrin* formation) but do greatly delay or inhibit the formation of true fibrin (the *coacervation* phase, according to Mommaerts) Some other agents such as urea guanidine etc, chiefly block the initial reaction which Mommaerts speculates may involve H bonds When an extremely small amount of thrombin is added to fibrinogen the viscosity immediately starts to increase and continues to rise Mommaerts attributes this to increased asym metry of the fibrinogen (? profibrin) molecules due to aggregation mainly or exclusively in a longitudinal (end to end) direction Mommaerts recently (1948) reviewed this work at a seminar in Dr O Neurath's laboratory at Duke University At that time I learned something about his methods and particularly the techni cal difficulties under which some of the cited experiments were carried out There seems little doubt that the validity of some of his data is questionable He was not informed for instance on the data of Seegers et al as to the probable molecular weights of highly purified thrombin and prothrombin He is especially to be criticized in his claim that the isoelectric point of thrombin lies in the far alkaline range I believe Dr Seeger's best figures for the isoelectric points are thrombin 4 4 prothrombin 4 8 (pH) accord ing to data on their precipitation range I do not know whether Dr Seegers used standard buffers and the photoelectric turbidi metric technique [*ref* J H Ferguson *J Gen. Physiol* 25 *607 (1942)*] which could establish the 'points of minimum solubility with considerable accuracy

In connection with the profibrin idea P W Boyles and I have recently repeated some of Apitz s experiments with the addi tion of certain data, e g fibrin yields and certain *control* tests (Table 10) not found in the German work The following is typical of the salt experiment A completely prothrombin free ($BaSO_4$ adsorbed) fibrinogen (about 0 5 per cent) was mixed (1) with a very weak highly purified (Seegers) thrombin solu- tion, in the presence of 4 5 per cent NaCl In the controls the fibrinogen (3) and thrombin (2) were incubated alone with the salt, the other agent being added with the water of dilution in the

With regard to the work of Glazko (*op cit*) begun in California under D M Greenberg and continued with us in Michigan I should like to recall the marked inhibitory effects of polyvalent anions on the clotting time of thrombin fibrinogen mixtures I note in one paper [*Am J Physiol* 134 *54 (1941)*] that 1/2048 molar potassium ferrocyanide detectibly increased the clotting time from 22 (control) to 25 (test) The following is quoted from Glazko s Ph D Thesis (op cit)

The amount of thrombin used to coagulate the fibrinogen makes a great difference in the apparent effect of the polyvalent anions When more thrombin is used more of the salt must be added to obtain the same degree of inhibition It was observed that when coagulation was completely prevented by the presence of sufficient polyvalent anion the addition of more thrombin would cause coagulation to occur

TABLE 11 *

$K_4Fe(CN)_6$ mols/liter	2	4	6	8	10 (rel thrombin conc)
0	15	10	7	6	5 (c t in sec at 28 C)
0 01	70	27	25	20	18
0 02	600	80	55	55	40
0 03	∞	∞	130	80	65
0 06	∞	∞	∞	∞	105

* *Table 14 of Thesis*

I would like to know what the physical chemists think of these data on the effects of polyvalent anions Is it just a matter of the availability of more charge or is it perhaps as Glazko suggests a matter of some rather specific effect on thrombin?

Recently E B Gerheim and I have been interested in staphylocoagulation Gerheim has proved incontestably that this mechanism is unrelated to ordinary thrombin clotting The following data (Table 12) are from Gerheim s *thesis* (1949)

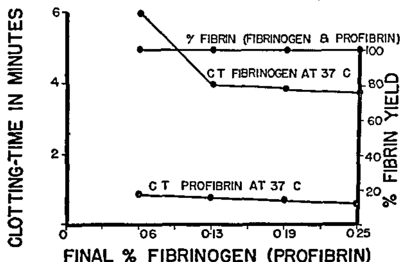


FIG 11

Note (1) the marked increase in reactivity (shorter clotting times) as the result of the heating but (2) the complete absence of effect on the (100 per cent) fibrin yields

among reactions of simple ions [V K. La Mer *Chem. Reviews* 10 *179 (1932)*] This is due to the reduction of attractive forces as I have described All that can be deduced on this basis is that the approach either of thrombin to fibrinogen or of two fibrinogen molecules to each other or both is influenced by Coulomb forces My remarks have illustrated one possible scheme for the operation of these forces

At equal molal concentrations potassium ferrocyanide has ten times the ionic strength of sodium chloride However this is not quite enough to account for the effects obtained by Glazko there are evidently specific ionic effects as well perhaps similar to those observed by Edsall and Lever with thiocyanate and iodide

Finally I have some comments on the work of Mommaerts His experiments with various electrolytes give further proof of the importance of electrostatic forces in the clotting process but, because the effect of ionic strength on rates of ionic reactions is a universal one (as pointed out above) his results do not provide any basis for assumptions about the structure of the clot. Mommaerts original concept of a coacervate involved a mosaic of positive charges contributed by thrombin and negative charges contributed by fibrinogen I believe This must be rejected first because we now know both proteins are negatively charged and second because the proportion of thrombin which suffices for clotting is so small (10^{-6} or less) that it cannot possibly make any structural contribution its role must be enzymatic. On the other hand a concept of a mosaic of positive and negative charges both contributed by the fibrinogen is similar to my own picture

The experiments of Laki and Mommaerts at pH 5 1 have worried me because of the absence of a control to show what happens to fibrinogen kept at pH 5 1 in the absence of thrombin If it is partly denatured it might well clot faster because of the fibrinoplastic effect which Dr Ferguson has described elsewhere. In any case I am not sure that the phenomenon observed in these experiments those of Apitz and those of Ferguson and Boyles (in which the proper controls were included) ought to be called a two-stage reaction Why not just say that the polymerization of fibrinogen has proceeded part way in the presence of the retarding agent (sodium chloride in the latter case) and therefore has less far to go when the retarding agent is removed? Instead of two stages there must be an infinite number of stages in the polymerization process as the fibrinogen molecules add on one by one I am not yet

TABLE 12

	Prothrombin free FIBRINOGEN	'Cofactor' (HUMAN SERUM ALBUMIN)	*Prostaphylo* coagulase	Calcium + thrombo-plastin	Clotting time (37 C)
1	+	+	—	—	∞*
2	+	+	—	+	∞
3	+	—	+	—	∞
4	+	—	+	+	∞
5	+	+	+	—	39 min

*The negative tests were followed over 4 days

No such clotting phenomenon could be obtained with streptococcal cul ture material with or without cofactor'

Details of the reagents and test methods are given in a preliminary publication [*Proc Soc Exp Biol and Med 66 525 (1947)*] Our conclusion must be that 'fibrin" can result from the action on fibrinogen of several apparently unrelated 'coagulants" It would be desirable to pursue these enquiries further with the aid of the electron microscope

Ferry I should like to comment on Dr Ferguson s remarks if I may before the discussion continues I will take them in reverse order

First the distinction he has drawn between true fibrin clots with a characteristic fibrillar structure and certain systems which may look like clots in casual inspection but lack the fibrillar network is an extremely important one It is clear that some coagula which may be obtained in experimental work under abnormal con ditions may have structures quite different from that of genuine fibrin

Second, the retardation of clotting by polyvalent ions could be explained in part as the well known effect of increasing ionic strength which decreases the rate of *any* reaction between mole cules carrying charges of opposite sign many examples are known

Smith It has to be built over?

Ferry Yes it has to be built over because collagen is full of cyclic amino acids which must alter the fundamental backbone structure of the protein

Smith A difference in morphology is hinted at by Bates and others

Ferry Astbury in England has pointed out that fibrinogen and myosin are very similar in some aspects of x ray spectra but collagen seems to belong to a quite different family

Flynn Do you consider profibrin a distinct entity?

Ferry I have heard the name profibrin used for an intermediate between fibrinogen and fibrin I think it is an unfortunate term because it does suggest that there is a distinct species whereas I think it cannot be doubted that there is a whole spectrum of intermediates ranging from a dimer of two fibrinogen molecules joined together to polymers of five ten one hundred and finally an infinite number of molecules which is the fibrin clot Profibrin probably represents a vaguely defined intermediate

Barker Is fibrinogen B also an intermediate?

Ferry I am not familiar with fibrinogen B

Knisely You spoke about the rate of turnover the special term thermal fusion

Ferry That is the rotatory diffusion constant

Knisely What about the effect of polymerization? What will two or three, or four molecules sticking together do what about the rate?

Ferry The rate will decrease with the cube of the length of the chain will it not Dr Edsall?

Edsall It would slow it down considerably It would be about the cube of the length it would slow it down by that

Quick There is one compound which I might suggest that Dr Ferry investigate and that is Bayer 205 or the old Germananin In my early work I obtained some indication that it exerts its inhibitory action on fibrinogen Whether that is correct or not I don t know

Jaques With regard to Dr Fremont Smith s mention of motion in the system experiments I did years ago on the conversion of fibrinogen to fibrin raise I think a point which has been overlooked In this work [*Biochem J* 32 *1181 (1938)*]

convinced that the early stages and the final stages differ in any fundamental way

Fremont Smith I would like to ask two questions Does the orientation of fibrinogen molecules account in some degree for the fact that blood is more likely to clot in stagnant or quiescent condition than when in motion? The other question—and this might be quite fantastic—it is possible to impose an electric field on a solution about to clot and thereby increase the speed of clotting of the previously oriented molecules?

Ferry Those are very interesting points I doubt, first, if any flow in the vascular system would have any effect on the orientation of individual fibrinogen molecules asymmetric though they are, it takes quite high velocity gradients to produce any perceptible orientation However, after the polymerization had proceeded to some extent so that there were trains of partially polymerized fibrinogen floating around it is quite likely that such aggregates could be oriented by flow

With respect to the electrical field unfortunately fibrinogen is only soluble in the presence of fairly substantial amounts of salt and it is impossible to impose an electrical field on a conducting solution which would be high enough to produce the orientation I fear In other words one would simply get conduction of current by the salt and the other molecules would not be involved I know that it would be impossible to impose sufficient potential because it would generate so much heat by the electrical conduction that the system would boil

Fremont-Smith Even with a prolonged current flow Dr Ferry?

Ferry It has to be a high potential and that is unfortunate because it would be a very interesting experiment

The thermal rotatory diffusion is sufficiently powerful to keep these things pretty well in a random state of orientation

Smith There has been much interest among histologists about the possible relationship between fibrin and collagen I wondered whether Dr Ferry had anything to say in a very few words as to whether this work fits with what is known about collagen, whether we are destroying or substantiating that theory

Ferry In the first place the amino acid compositions are very different so I don't believe that it is really possible to draw a conclusion concerning one from the other

However there is the possibility that other analytical data might give us information For one thing, there are several reports for methionine values for fibrin fibrinogen (both our own and those of Brand et al) It is rather interesting that the methionine value for fibrin is slightly higher than the methionine value for fibrinogen and some values I obtained some years ago for the carbohydrate content of fibrinogen and fibrin again showed a slight increase (fibrinogen—2 64%, fibrin—2 78% carbohydrate) These values were based on the ratio of carbohydrate to nitrogen I wonder whether that indicates a decrease in some other constituent of the protein Of course there are definite difficulties in the consideration of the analytical data such as the difficulty of preparing both fibrinogen and fibrin dry and salt free, to give the same basis of comparison for the analytical data on the two proteins

Dr Ferry mentioned cysteine and the question whether a modification of fibrinogen would affect the conversion of the fibrinogen to fibrin We found that oxidation of methionine in the fibrinogen would still allow the demonstration of the conversion of fibrino gen to fibrin [*Biochem J* 37 *344 (1943) Can J Research* E24 *79 (1946)*] I suggest that further studies of other groups might add some information in this regard

Wright Dr Ferry there are several of these points I think you would like to discuss

Ferry I believe it is unlikely that a single hydrogen bond or even a few hydrogen bonds are strong enough to bind the fibrinogen molecules together Only a very large number of hydrogen bonds which would involve a high configuration of one molecule fitting against a complimentary configuration on the other would give the necessary stability I would agree with Dr Jaques that nothing is added when one proposes that clotting is denaturation but fails to explain what kind of denaturation Often denaturation of so-called corpuscular proteins is supposed to involve an unraveling of the polypeptide chain which forsakes its compact ordered arrangement for some sort of open random coil I feel it is very unlikely that this sort of intramolecular arrangement is involved for several reasons In the first place x ray diagrams of fibrinogen and fibrin briefly described by Bailey and Astbury several years ago were reported to be practically identical which could hardly be the case if a profound intramolecular arrangement had taken place In the second place usually when a protein with the amino

the study was carried out with rocked tubes just the same as enzyme chemists carry out their investigations, rather than with the procedure normally used by workers in the clotting field, who allow the tube to stand still and the gel to form. I think this explains the fact that there were some interesting points in kinetics in that paper The initial velocities showed the same relationship to enzyme concentrations as you find with any of the systems more commonly studied by the enzyme chemists I think this is a technique which is of value and sometimes gives information which we are apt to overlook if all the studies are conducted from the standpoint of just allowing a complete gel to form

I was very interested in Dr Ferry's presentation The problem still remains as to what is the nature of the activation process brought about by thrombin and in turn, what are the actual bonds involved in the bonding I wonder if Dr Ferry would make any comment on the work of Laki [*Z physik Chem* A190 *278 (1942)*] who found a difference between fibrinogen and fibrin on the basis of a new absorption band developing at 400 mμ with fibrin Laki *concluded this represents hydrogen bonding of nitrogen to oxygen* I mentioned last year that we have been unable to confirm Lyons work of tracing the linkage to SH groups and are still of the opinion that it does not involve the SS linkage As Dr Ferry recalls Wohlisch some years ago suggested the action of thrombin fibrinogen was that of denaturation only in this case, produced by an enzyme instead of one of the ordinary agents of denaturation That does not tell us very much except to give us another word for the process There are some definite points though that are contrary to this view There is a definite difference in digestibility by fibrinolysin between denatured fibrinogen and fibrin A point that I have observed with the nitroprusside reaction is that fibrinogen will only give an indirect positive nitroprusside reaction after denaturation and the same can be shown for fibrin that is if you take fibrin freshly prepared it does not give the nitroprusside reaction *before denaturation any more than fibrinogen*

One point that I would like to suggest—and Dr Ferry has some fairly good ideas on it—is that I believe we still lack a good comparison of fibrinogen and fibrin from the standpoint of analytical data *I made the point some years ago (and at that time of* course, it was of some significance because of the early theories that thrombin broke down fibrinogen to fibrin plus some other protein) that all the nitrogen of fibrinogen can be recovered as fibrin

Ferry With respect to the study of high pressures there has been some work at Princeton on the effect of high pressures on other enzymatic reactions [F H Johnson *Adv Enzymology 7 215 (1947)*] The inhibition by high pressure has been interpreted by Eyring in terms of activated complexes formed with the enzyme If there is a volume increase in the formation of the activated complex, *then the presence of high pressure will shift the equili* brium away from the activated complex That just follows from Le Chatelier's principle Perhaps the same thing will hold here if so it will be concerned with the reaction between thrombin and whatever group on the fibrinogen molecule is specific Do you know whether these effects were reversible?

Seegers I don t recall at the moment but I believe that they were reversible

Ferry That I think would correspond to the phenomena treated by Eyring

Fremont Smith Is there a volume increase with fibrin formation?

Ferry I doubt very much whether this could be measured without extremely delicate apparatus

Fremont Smith Nothing then can be done?

Ferry I don t think so In the work with other enzymes—concerned with bacterial luminescence—the volume increase was inferred and not measured

Quick The reference Dr Seegers mentioned is one by Ebbecke and F Knuchel [*Arch f d ges Physiol* 243 *pp 43 and 65 (1939)*] I think there are several other rather involved articles in this series all of which appeared in the aforementioned journal

Edsall With respect to the amino groups I believe that the effect of nitrous acid on a protein molecule may be something more far reaching than just deamination of the free amino groups I should like very much to see Dr Seegers try acetylation of the amino groups of purified thrombin with ketene in the same way that Northrop and Herriott did it on pepsin In that case they could acetylate all the free amino groups and the enzyme is still active When they acetylated the tyrosine hydroxyl groups the activity of the enzyme decreased progressively but activity could be restored by splitting off the acetyl groups

In principle the same study could be done on thrombin It might really give us some significant answers with respect to the

acid composition of fibrinogen—that is, containing a fair number of hydrophobic groups—is denatured, it becomes insoluble The hydrophobic groups are exposed and the molecules ball up into a compact mass, this is not the case in the fibrin clot In the third place it seems very unlikely that a loose random coil would ever be able to fit itself into those precisely aligned fibers so I think the case against an unraveling type of denaturation is good

With respect to chemical analysis I think it is extremely important that purified fibrinogen and fibrin be subjected to analysis not only for the usual amino acids but perhaps for some other odd things which might be there in small amounts unsuspected It is quite likely that the values are very low and because of the high molecular weight would require fine analysis Brand and his associates have performed an analysis on fibrin and fibrinogen but it was not fine enough to have meaning from this point of view

Ferguson I would like to know if Dr Ferry or any member of the group would comment on the claims of Laki to have crystallized fibrinogen It seems to me that that is a very extravagant claim and there is so much chance that the material so obtained was not fibrinogen

Edsall We once made an attempt to repeat his work and did not succeed in getting crystals We tried several times without results and I know of no one else who has obtained them I should suspect that what he got was either not fibrinogen or not crystalline

Seegers I wonder if Dr Ferry would care to comment on several questions? One of them deals with the observation recorded in the German literature—I don t remember the author—that at high pressures (several atmospheres) thrombin will not clot fibrinogen The second question has to do with the fact that we have been able to demonstrate a platelet material which accelerates the interaction between the fibrinogen and thrombin Would you venture an opinion as to how this acts?

A third question—I doubt that it should be directed at Dr Ferry Perhaps it should be asked of Dr Edsall I believe I heard him say he has some doubt as to whether free amino groups have anything to do with the interaction between fibrinogen and thrombin I have treated thrombin with weak solutions of nitrous acid and after you get all through with that it will not clot fibrinogen I wonder whether nitrous acid is attacking the free amino groups or whether something else is altered

Jaques That could mean that any two groups of fibrinogen were responsible for the enzyme substrate combination yet an adjacent group would be the one activated Would that be possible?

Edsall Several different kinds of groups may be essential to the reaction I think

kind of treatment the molecule could undergo and the nature of the groups that are essential for activity At present, we are still very much in the dark I know that Dr Seegers has very little of his purified thrombin and it may be impracticable to carry out the experiments now

Jaques I thought your original reference was to the amino groups of fibrinogen

Edsall I was thinking more of the amino groups of fibrinogen than of thrombin What I had in mind was Chargaff's work on these artificial coagula which may or may not resemble true clots Studies with the electron microscope may be decisive in indicating whether genuine clots were produced by naphthoquinone and ninhydrin derivatives Chargaff did get some indication of the involvement of amino groups in these reactions I have forgotten the details

May I make one remark about the work of Mommaerts? He made one remark about electrostatic interaction between fibrinogen and thrombin and referred to some unpublished work of his own indicating the thrombin had quite an alkaline isoelectric point I have seen no confirmation of that work and it stands in direct contradiction to the work of Dr Seegers

Ferguson He recently explained that that work was crude and not confirmed and he is now willing to give up that idea as an error

Ferry It seems highly probable that electrostatic interaction between thrombin and fibrinogen is involved in bringing the thrombin to the right spot on the fibrinogen molecule for its activation If thrombin carries a negative charge in the physiological pH range as I gather it does then we may infer that the group on the fibrinogen molecule it attacks is on a positively charged region

Edsall After all two ions can interact even if they both carry a net charge of the same sign provided one is a protein containing a large number of both negative and positive charged groups For instance serum albumin even at pH 7 or above when it carries a large negative net charge can still combine with a large number of anions The work of such men as Bennhold Klotz Luck, Scatchard and others has shown that quite conclusively

anticoagulant power of a surface was inversely proportional to the force of adhesion between that surface and water or in other words the wettability of the surface J S Hirschboeck [*Proc Soc Exper Biol & Med* 45 *122 (1940)* 47 *311 (1941)*] has reported on delayed coagulation times when blood was tested in methyl methacrylate (boilable lucite) tubes and in collodion lined tubes He found that collodion as a surface did not follow Lampert s law since the force of adhesion of water on a collodion surface was greater than that on a paraffin surface but the coagulation times on a collodion surface were longer than those on a paraffin surface However the differences were not great nor entirely consistent and Hirschboeck drew the blood into uncoated glass syringes before transfer to the coated tubes

In 1942 E L Lozner and F H L Taylor [*J Clin Investigation* 21 *241 (1942)*] determined the coagulation times of blood in tubes made of a plastic material called lusteroid and found them longer than when glass tubes were used A H Kadish [*Am Heart J* 34 *212 (1947)*] working in our laboratory in 1944 also did tests in lusteroid tubes In 50 normal subjects he found that the coagulation times varied from 14 to 28 minutes with a mean of 19 as compared to times of 4 to 10 minutes with a mean of 6 when portions of the same samples of blood were tested in glass tubes He withdrew the blood from the vein into oiled glass syringes He also made tests on patients with intravascular thrombosis In 8 of 11 cases of acute thrombophlebitis the coagulation time in lusteroid tubes was less than 14 minutes although the coagulation time of these same patients in glass tubes was within normal limits Kadish did all his tests at room temperature Unfortunately he found that the coagulation times varied considerably with different lots of lusteroid tubes and was forced to conclude that either the composition or the surface of the lusteroid might vary considerably in the process of manufacture

In 1946 L B Jaques E Fidlar E T Feldsted and A G MacDonald [*Canad M A J* 55 *26 (1946)*] reported on the use of one of the silicones (methyl chloro-silane) a liquid manufactured by the General Electric Company and labeled by them Dri Film No 9987 This silicone can be applied to the surface of glass tubes glass syringes and metal needles and deposited as a very thin but apparently complete coating by rinsing the surfaces with distilled water and then a dilute solution of ammonium hydroxide The surface is slippery and relatively nonwettable

SURFACE EFFECTS ON BLOOD COAGULATION

NELSON W BARKER and HAROLD MARGULIES

Division of Medicine Mayo Clinic

Several variables may influence the coagulation time when whole blood, to which no anticoagulant has been added, is tested to determine how long it takes for coagulation to occur after it is removed from the vascular system Among these variables are the environmental temperature the size of the container in which the blood is placed the amount of blood which is used for the test the rapidity and degree with which the container is tipped to determine the end point, and the amount of tissue thromboplastin which may be introduced into the blood during its withdrawal from the vascular system We suspect that there may be variations in the amounts and behavior of the various elements of the blood itself among different individuals Another variable is the character of the solid surface of the containers in which the blood is collected and tested

Most standard tests of the coagulation time of whole blood or plasma have been made in glass containers because glass is cheap available and transparent, and this transparency permits good appraisal of the beginning and the end of the gross coagulation process However, even glass varies in regard to its chemical com position and the character of its surface Many years ago, E Freund [Quoted by J Bordet and L Delange *Ann Inst Pasteur* 26 *657 (1912)*] showed that blood will clot much more slowly in a glass tube if the tube is first coated evenly with paraffin O Inchly [*J Pharmacol & Exper Therap 18 237 (1921)*] noted that when the clotting test was done on wire loops the type of metal and its cleanliness affected the coagulation time In 1931 H Lampert [*Die physikalische Seite des Blutgerinnungsproblems und ihre praktische Bedeutung* Leipzig Georg Thieme 1931 127 pp] reported on an extensive study of the effects of surface on the coagulation time Lampert reported variations in the coagulation time when the blood was tested in containers made of glass, quartz, various metals and plastics and found that athrombit, a plastic made from one of the resins had the greatest ability to delay coagulation Normal coagulation times in athrombit tubes at 37 C averaged 30 minutes Lampert s studies indicated that the

Immediately after the silicone tubes were filled from the silicone-coated syringe 1 cc of blood was placed in a glass tube Ten minutes after venipuncture 1 cc of blood was placed in another glass tube and this procedure was repeated twenty minutes after venipuncture also The glass tubes were kept at room temperature and tipped at intervals of one-half minute until coagulation was complete in these tubes

Tests were made on 5 normal persons at three different times on the same day, 7 normal persons on each of three different days at intervals of several weeks and on a total of 50 normal persons altogether Single tests were also made on 27 patients who had acute intravascular thrombosis Results are shown in Tables 13 to 16 Although the total number of patients with thrombosis is small and the results would be more significant if more tests had been made on each of these patients we believe that a definite trend is indicated There is no significant difference between the coagulation times in the glass tubes at room temperatures of the normal subjects and of patients who have thrombosis (Table 13) The coagulation times of blood of normal persons in the silicone tubes were markedly prolonged over those in the glass tubes—a mean of 38 6 minutes as compared with a mean of 11 7 minutes (Tables 13 and 14) There is a tendency to shorter coagulation times in silicone tubes among the patients with acute thrombosis

TABLE 13

COAGULATION TIMES IN MINUTES OF WHOLE VENOUS BLOOD IN GLASS TUBES AT ROOM TEMPERATURE

	Number	Coagulation times in minutes			
		Range	Mean	Standard deviation	Standard error of mean
Normal subjects	50	5—19	11 7	2 85	0 40
Patients with acute thrombosis	27	8—19	11 9	2 68	0 52

Jaques and his co workers found that the coagulation time of blood drawn into silicone coated syringes through silicone-coated needles and transferred to silicone-coated tubes was markedly delayed as compared with tests done in uncoated glass tubes If kept at 20 C blood remained fluid for from one to several hours When blood was transferred from a silicone coated container to a glass tube fifty three minutes after venipuncture the clotting time in the glass tube was not reduced Plasma stored six weeks in silicone-coated containers in a refrigerator remained fluid and then when transferred to glass at room temperature, clotted in the normal time Many of the members of this conference have used the silicone technique in studies of coagulation since the method was reported by Jaques and his associates It appears that silicone delays the coagulation time of blood when it is outside of the vascular system more than any other surface studied as yet

We have made a number of tests of coagulation time on whole blood in silicone coated apparatus with the technique outlined by Jaques and his co workers We were interested chiefly in determining whether the coagulation times of the blood of patients who had intravascular thrombosis were significantly shorter than those of normal persons The tests were made on whole blood withdrawn from the antecubital vein by a clean and quick venipuncture into a silicone coated 10 cc syringe Eight to 10 cc of blood were withdrawn and tests were made in test tubes 7 cm long with an internal diameter of 8 mm One cubic centimeter of blood was placed in each of three such tubes which had been coated with silicone These tubes were immediately placed in a water bath which had a temperature of 37 C One tube was tilted at intervals of five minutes until it was noted that the coagulation process had begun It was then tilted at intervals of two minutes until coagulation was complete and the clot remained in the end of the tube after inversion The time when this occurred after venipuncture was considered the end point When the end point occurred in the first tube the second tube was tested every two minutes until the end point was reached and then the third tube was tilted every two minutes until the end point was reached The coagulation time was almost always a little longer in the second tube than the first and in the third tube than the second This probably indicated the accelerating effect of more frequent tipping The coagulation time in the third tube was arbitrarily chosen as the significant time for that particular sample of blood

had silicone-coagulation times of 30 minutes or more as compared with only 20 per cent of those with acute venous thrombosis This seems to indicate that the blood of many of the patients with acute venous thrombosis has some tendency toward increased coagulability The tendency cannot be detected when the test is done in a glass tube but may be detected when the test is done in a silicone-coated tube It is not certain whether or not this is a transient tendency since the blood of these patients was not tested before thrombosis occurred nor at various intervals after thrombosis occurred

A second point noted by Jaques and his associates is indicated in Table 16 in which coagulation times were determined after transfer from the silicone-coated syringe to a glass tube at intervals Little change occurred in the coagulation time when the transfer was made ten minutes after venipuncture as compared with the time when the transfer was made immediately after venipuncture However, the coagulation time shortened appreciably when the transfer was made twenty minutes after venipuncture This finding indicates that some phase of the coagulation process had probably already started in the silicone coated syringe This suggests that at room temperatures during the first ten minutes after withdrawal when blood is in contact with silicone there is probably

TABLE 16

COAGULATION TIMES IN MINUTES OF WHOLE VENOUS BLOOD COLLECTED IN SILICONE COATED SYRINGES AND TRANSFERRED TO GLASS TUBES

Time of transfer after venipuncture	Number	Coagulation times in minutes			
		Range	Mean	Standard deviation	Standard error of mean
Immediately	50	5—19	11 7	2 85	0 40
10 minutes	46	6—20	10 6	2 95	0 44
20 minutes	46	2½—16	7 5	2 80	0 41

TABLE 14

COAGULATION TIMES IN MINUTES OF WHOLE VENOUS BLOOD IN SILICONE COATED TUBES AT 37 C

	Number	Coagulation times in minutes			
		Range	Mean	Standard deviation	Standard error of mean
Normal subjects	50	25—57	38 6	8 20	1 16
Patients with acute thrombosis	27	12—53	29 2	11 27	2 13

TABLE 15

COAGULATION TIMES IN MINUTES OF WHOLE VENOUS BLOOD IN SILICONE COATED TUBES AT 37 C

	Total number	Coagulation times in minutes					
		12 to 24		25 to 29		30 or more	
		No	Per cent	No	Per cent	No	Per cent
Normal subjects	50	0	0	6	12	44	88
Patients with acute venous *thrombosis*	20	11	55	5	25	4	20

than in the normal subject This is indicated in Table 14 and even more significantly in Table 15 where from the group of 27 patients with acute thrombosis only those who had acute venous thrombosis were selected and the number who had silicone-coagulation times of *from 12 to 24 minutes 25 to 29 minutes and 30* minutes or more are compared For example none of the normal group had coagulation times of less than 25 minutes whereas 55 per cent of those with acute venous thrombosis had silicone coagulation times in this range Also 88 per cent of the normal patients

needles into silicone-coated tubes 16 mm in diameter They found that with the prothrombin activity only 5 to 15 per cent of normal there was no effect on the coagulation time in glass but that the coagulation time in silicone was often 24 hours or longer Their normal silicone-coagulation time was 30 to 60 minutes We also tested the effect of dicumarol on the coagulation time of whole blood in glass tubes and silicone-coated tubes and on the one-stage prothrombin time of the same samples of blood The changes in the silicone coagulation times from day to day for 13 patients who were receiving therapeutic doses of dicumarol were similar but less marked than the changes in the prothrombin times Two sample sets of curves are presented (Figs 12 and 13) We found little correlation when all the prothrombin times of these patients were compared with all of the silicone-coagulation times How ever when the curves of each individual patient were compared a definite tendency toward parallelism was found in the rise and fall of the silicone-coagulation time and the prothrombin time A lag of a day or so usually occurred before a rise or fall in the prothrombin time which was followed by a similar rise or fall of the silicone-coagulation time Thus while usually no change can be demonstrated in the coagulability of whole blood of patients who are receiving therapeutic doses of dicumarol if the test is done in a glass tube the more sensitive test in a plastic or silicone-coated tube does show definite changes

The question may be asked as to whether or not the silicone-coagulation time might not replace the one-stage prothrombin time as a guide to the administration of dicumarol since it does not require the use of thromboplastin Unfortunately the silicone coagulation time tests are time-consuming particularly on patients receiving dicumarol It takes some time to prepare the syringes and tubes and we cannot recommend the test as having very accurate reproducibility even when considerable time is spent in learning the technique The range of times for normal individuals is considerable and we doubt that critical levels could be established even with an enormous number of tests With the one stage pro thrombin time test once the setup is made and the technique is learned many tests may be done in a relatively short period of time with considerable accuracy and reproducibility for that par ticular laboratory

Unfortunately we have no data indicating a similar compari son between the effects of dicumarol on the silicone coagulation

little change in the coagulation mechanism and that the coagulation process may begin sometime between ten and twenty minutes after venipuncture It also suggests that, if the blood is kept in silicone coated containers at room temperature after being withdrawn into a silicone coated syringe there is a period of between ten and twenty minutes during which it may be handled for experimental purposes without the addition of any anticoagulant and without the coagulation mechanisms being initiated This interval is shorter than that found by Jacques and his co-workers They stated that their room temperatures were 20 C Our tests were done at room temperatures of 23 to 25 C There may have been other differences in fine points of technique between the two series of experiments

It has been fairly well established that the coagulation times of whole blood in glass tubes do not vary significantly from the normal among patients who have received therapeutic doses of dicumarol even though there has been a significant elevation of the one stage prothrombin time The coagulation time of whole blood in glass may be moderately prolonged if the dose of dicumarol has been excessive, and the prothrombin time is greatly prolonged beyond the so-called therapeutic range For this reason coagulation times of whole blood in glass containers have not been of any value in the determination of the effect of dicumarol unless this effect was excessive C S Davidson and H A MacDonald [*Am J Med Sc* 205 *24 (1943)*] reported that when coagulation times were done in lusteroid tubes, the times were often prolonged when there was only moderate deficiency of prothrombin activity and when the coagulation time in glass was not affected

In his work on the coagulation times of blood in lusteroid tubes A H Kadish [*Am Heart J* 34 *225 (1947)*] studied some patients who were receiving dicumarol He found that the coagulation times in the lusteroid tubes were usually increased to some degree when the prothrombin time was increased although there was a tendency for the effect on the lusteroid coagulation time to lag behind the effect on the prothrombin time in any one patient W C Moloney A. S Murphy and W J Harrington [*Am J Med* 5 *40 (1948)*] have reported on simultaneous studies of coagulation times at 37° C in silicone coated tubes and glass tubes and prothrombin times of 11 patients who were receiving dicumarol They found that the silicone-coagulation time roughly paralleled the prothrombin time They collected blood directly from uncoated

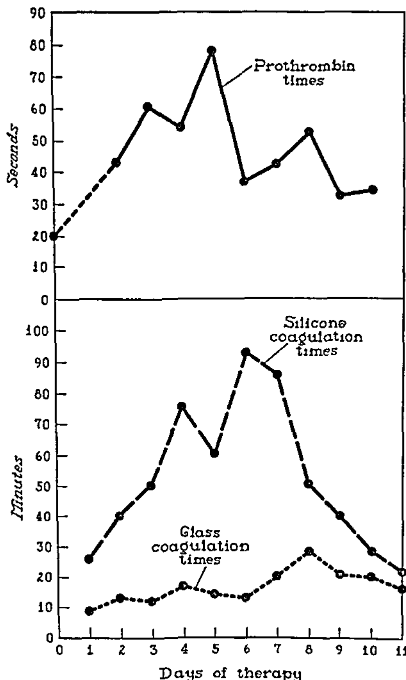


Figure 13 — Effect of oral administration of dicumarol on coagulation time of whole venous blood in a glass tube whole venous blood in a silicone-coated tube and one stage prothrombin time of plasma of a human being

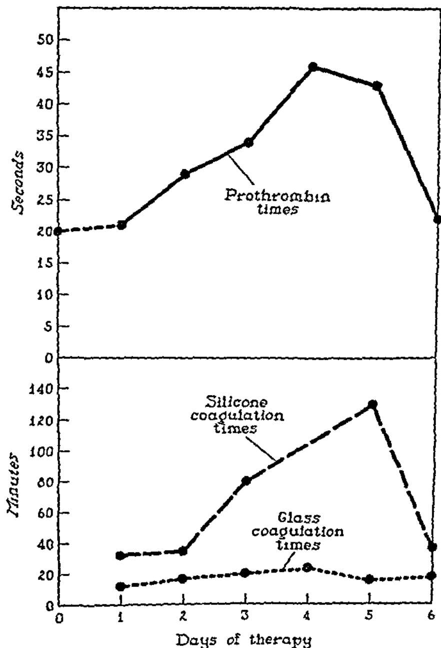


Figure 12 — Effect of oral administration of dicumarol on coagulation time of whole venous blood in a glass tube whole venous blood in a silicone-coated tube and one stage prothrombin time of plasma of a human being

F D Mann M Hurn and D R Mathieson (unpublished data) have recently studied the behavior of platelets during the clotting of plasma which had been in contact only with silicone surfaces and to which anticoagulants had not been added A small drop of this plasma was transferred to a silicone-coated glass slide and covered with a silicone-coated glass cover slip which was sealed with petro latum The preparation was studied under the phase contrast microscope with which the structure of platelets and fibrin were sharply visualized As distinguished from preparations of oxylated plasma in contact with glass surfaces agglutination of platelets was absent in the silicone preparation and there were initially very few cytoplasmic processes Such processes sometimes five times as long as the diameter of the platelets as illustrated by A Fonio and J Schwendener (*Die Thrombozyten des menschlichen Blutes und ihre beziehung zum gerinnungs—und thrombosevorgang* Bern Hans Huber 1942 130 pp) did develop but appeared more tenuous than those seen in glass preparations The platelets and processes were in constant Brownian movement Fibrin needles first appeared about an hour after the blood had been drawn The first needles were not connected with the platelets but soon connections formed the Brownian movement ceased and the processes no longer could be seen Additional threads of fibrin became attached to the platelets and seemed to become thicker at these attachments

It is difficult to escape the conclusion that the delayed effect of coagulation in silicone as compared with glass is at least in part due to the difference in behavior of the platelets which by comparison do not agglutinate or adhere to the silicone develop their cytoplasmic processes much more slowly and therefore prob ably liberate their factor which takes part in the development of thromboplastin much more slowly

In summary it can be stated that the coagulation time of whole blood may vary considerably depending on the surface of the containers into which it is shed or drawn when other factors are kept as constant as possible Silicone appears to be a type of sur face which produces as much or more delay in the coagulation process as any type of foreign surface which has yet been tried Even when all variables are controlled as well as possible silicone coagulation times of whole blood at 37 C may vary appreciably when determined on different portions of the same sample of blood on samples from the same person drawn at different times of a

time and the two stage prothrombin determination This might be interesting because frequently the effect of dicumarol on the two stage prothrombin test lags behind its effect on the one stage test just as does the silicone coagulation time

As has been indicated in other studies, the effects of dicumarol on the coagulation mechanism may not be confined to the effect on prothrombin alone but also may involve the platelets, the fibrinogen and other factors Thus, it is probable that the silicone coagulation time indicates the sum of the effects of dicumarol on all the coagulation factors of the blood, and if the test could be reproduced with a little more accuracy and less variability among different individuals, it might be a better test of the effect of dicumarol on the overall coagulation tendency than the one stage prothrombin time

The exact way in which different surfaces vary the speed of the coagulation mechanism is speculative Certainly some surfaces are grossly rougher than others, and it can be easily determined that some are more wettable than others As compared with silicone and various plastics glass is certainly rougher and more easily wettable J Bizzozero [*Virchows Arch f path Anat* 90 *261 (1882)*] expressed the opinion that, when platelets and leukocytes came in contact with surfaces other than the endothelium of blood vessels they became disrupted and liberated thromboplastic material It has been stated also that certain colloids change their behavior when they come in contact with foreign surfaces J W Pickering and D H de Souza [*Biochem J* 17 *747 (1923)*] suggested that fibrinogen had a colloidal protective coat which was removed somewhat slowly in paraffin coated containers but was rapidly removed when it came in contact with glass R A Gortner and D R Briggs [*Proc Soc Exper Biol & Med* 25 *820 (1928)*] on measuring electric charges were of the opinion that the platelets were positively charged and therefore were attracted much more rapidly to glass surfaces having a relatively high negative charge which was not possessed by plastics paraffin and silicone L M Tocantins [*Proc Soc Exper Biol & Med* 57 *211 (1944)* and *Am J Physiol*, 143 *67 (1945)*] has demonstrated the presence of anticephalin an antithromboplastic substance in the blood and has shown that when this substance is removed from plasma the clotting mechanism is accelerated He further demonstrated that glass absorbs anticephalin more rapidly than other surfaces such as lusteroid and paraffin

Ferguson I am thoroughly familiar with this set of appearances and we described them very carefully under the dark field in [*Am J Physiol* 108 *670 (1934)*] I have had occasion also to use the phase microscope on them more recently and would say the phase microscope is quite helpful in revealing them in comparison with the ordinary microscope but I think the dark field is superior

There are many interesting questions that arise in connection with surface factors in blood coagulation I think Dr Barker has made it interestingly clear that one must start with the surface phenomena in relation to the formed elements of the blood I should like to take up a little time with that topic first and then perhaps say something about surface phenomena in relation to the colloidal systems of the plasma In using the silicone technique in connection with platelet observations Dr Barker has added some new data Platelet alterations were carefully studied by Aynaud in 1909 [Le globulin des mammiferes These de Paris] We became interested in this topic about 1929 30 For the slides described here see the following two publications [*(a) Am J Physiol* 108 *670 (1934) (b)* J Elisha Mitchell *Sci Soc* 61 *148 (1945)*] (Slide *(b)* III 1) This is an oil immersion photomicrograph under the dark field microscope of the platelets in citrated rabbit blood plasma obtained by slow centrifugation Clotting is in abeyance and the platelets show the typical stellate forms There are many minor modifications of this (*a*) but the essential phenomenon is the appearance of tiny filiform excrescenses sticking out from the sides of the platelet It is possible immediately after the centrifugation to catch a few platelets before any significant alteration and these appear as little oval *discs* moderately refractile and with a few bright granules in their center

(Slide) This second slide is not in our publications but is something with which we are all very familiar namely the appearance of platelets in the ordinary Wright-stained blood film I should like to point out one particular platelet much larger than the others with a fairly definite oval outline and a rather diffuse staining of the azurophil granules

(Slide (*b*) III 3) The next slide is a dark field of an ordinary drop of human blood clotting on the microscope slide One or two single platelets are here seen in the altered form Around the

day or on different days, and among different normal persons Silicone coagulation times have been found in some patients with acute intravascular thrombosis to be shorter than the shortest normal times Whole blood may be kept in silicone containers for at least ten minutes at temperatures of 23 to 24° C without apparently initiating the coagulation process Although the coagulation time of whole blood in glass usually is not prolonged by administration of dicumarol, the coagulation time of whole blood in silicone is prolonged roughly but not absolutely proportional to the prolongation of the prothrombin time The exact reasons for the variable effects of different surfaces on the coagulation time are uncertain and may be the variable influences of the surfaces on platelets soluble fractions of the plasma or both

Wright Those processes actually grew out under vision and were not there originally?

Barker A very few of the platelets had small processes at the beginning of the period of observation but in most of the platelets the processes developed and grew while the platelets were being observed

Wright Did they grow from short to long or is it possible that they became visible because their rate of motion subsided?

Barker The processes were seen and grew while the platelets were in Brownian movement The movement did not cease until the fibrin strands had formed and attached themselves to the platelets The processes appeared to be very fragile and grew much more rapidly when blood was exposed to glass than when it was exposed to silicone One wonders if the processes may not be broken off when blood is subjected to high speed centrifugation and thus remain in the plasma which is relatively free of the platelets One also wonders whether the processes themselves may contain some of the thromboplastin activator or component of thromboplastin which is present in platelets and therefore may be the source of small amounts of thromboplastin in platelet free plasma

The platelets which had small processes at the beginning of the period of observation may have been old platelets It is probable that platelets like other formed elements of the blood go through various stages of involution and disintegration while circulating in the blood stream

of excrescences appeared in citrated blood whereas vesiculation appeared on recalcification, we believed this to be a significant clue as to the nature of the phenomena It is recalled that calcium salts reverse the phase relations in alkali soap suspensions On observing the edge of a film of phospholipid—we tested lecithin in (*a*) and cephalin and some others in (*b*)—the dark field affords a pretty demonstration of the phenomenon of myelin figure formation (R Virchow 1854 J B Leathes 1925)

(Slides (*b*) V, 12A 12B) These two slides illustrate this, in the case of lecithin With cephalin the tiny vesicular' forms of myelin figures are remarkably like the platelet alterations Further, under the dark field it is seen that not only do the fatty myelin figures flow *out* into the contacting watery phase but a corresponding penetration of water into the lipid phase also occurs When calcium salt is dissolved in the water the inward penetration (water into lipid) is much more striking We therefore believe these comparative studies throw much light on the fundamental mechanisms of platelet alterations In summary, these are principally myelin figure formations due to contact (surface) effects in which calcium salts play a specific role in directing the water into the lipid thus producing vesiculation and a limited degree of rupture (contributing platelet material to the surrounding fluid no doubt) It may be that certain types of *phospholipid,* e g cephalin are more important than others

We should however not lose sight of the fact that the platelet contains *protein* also and these studies do not preclude some change in protein about which the techniques of observation cited are unable to give any information

(Slides (*b*) IV 6-8) here illustrate very similar phenomena (photomicrographs under dark field) particularly of the vesicular excrescence type at the edges of mammalian megakaryocytes and of the thrombocytes of lower animals I think we should be a little cautious in jumping from these interesting observations to definite views about the origin and genetic relationships of the platelets a topic I shall return to in a minute But first let me point out support for the idea that phenomena of the type I have described are of still wider significance in the whole field of cell alterations under abnormal in vitro conditions

(Slide (*b*) V 10) This slide prepared by P H Ralph at Ohio State University is a striking illustration of filiform excrescences

central cluster of refractile granules are a series of little "vesicu lar" excrescences in some of which (or perhaps at their surface) are a few granules in vigorous Brownian movement Other platelets are in aggregated clumps, in which the alteration phenomena have occurred There are, *incidentally, numerous erythrocytes one* granular leukocyte, and some fibrin needles in the field

(Slide (*b*) III 2) On recalcifying the citrated plasma of III, 1, the platelets alter in a typical manner the filamentous excrescences and often the platelet as a whole quickly assuming the vesicular appearance Coincidentally fibrin formation occurs in the plasma but is *never significantly* at the platelet surface although the alter ing platelets become tangled up in it

(Slide (*a*) 1) Summarizing on this slide the typical sequence of *alterations* which the platelets undergo in shed blood, we note the following The unaltered circulating form is a little disc (described above) Seen edgewise especially if one or two filiform excrescences have begun to form it has the appearance of what Aynaud (1909 op cit) called batônnet * When the platelet disc comes into contact with a wettable surface (the changes being much retarded if such is avoided), it adheres, swells into a disc and then may continue spreading out on the surface It is this spreader form which we were looking at in the Wright stained film From this point most of the platelets form the curious little ' excrescences" previously noted Some of these are short and club like other long and filamentous and the most significant type are 'vesicular' in appearance These persist for a remarkably long time under prolonged observation with the dark field micro scope Usually, they stay attached or in close relation to the granu lar central mass whether of single platelets or the clumps into which many of them aggregate during the adherence and swelling phases Some excrescences do become detached however The semi rigid filamentous forms when free and oscillating under Brownian movement have been mistaken for spirochetes by a number of observers In noting that only the filamentous type

*Hal Downey in his Handbook of Hematology mistakenly translates this as little baton Actually the word refers to a child s home made toy simply fashioned by pointing one or both ends of a little stick used to play the game which the English call 'tip cat and the Boer children of South Africa kennetje The pointed end enables it to jump into the air when struck with a larger stick. While in the air it may be hit to as great a distance as possible and the distance paced off to make a score in the game

process of multipolar mitosis. It resembles certain cells in the testis and some pathological giant cells, especially those appearing in certain neoplasms. The resulting nucleus is one large fused multilobed mass. The important additional feature is that the cytoplasm of such cells is abnormal. In general terms, such a cell is ill-adapted to survive and it may be termed "necrobiotic," a word in common usage in pathological histology. It is because this is a dying cell that its pseudopods break away and further fragment to form platelets (J. H. Wright). It is exceedingly difficult to observe true platelet formation in isolated megakaryocytes. I failed to find this in numerous observations over several years and know of only two accounts of it in the literature (one by Florence Sabin). It was Levy's similar non-success here which led him back to the long defunct "artefact theory" of the blood platelets. The significant evidence however lies in the sectioned material of Wright and those who have confirmed him, together with the indirect support of platelet and bone-marrow studies in clinical conditions of thrombocytopenia and related purpuras [ref Chap XV M. Wintrobe's *Clinical Hematology* 2nd ed., (1946)]

I shall stop here and return later to the question of surface action in relation to the colloidal phenomena of clotting

Wright Dr Barker's, Dr Ferry's and Dr Ferguson's comments are open for discussion Are there questions regarding clotting time determination in silicone glassware?

Brinkhous Particularly when the clotting time is greatly prolonged, we have had great difficulty in selecting end points. I notice Dr Barker that you had some rather definite figures for the clotting time determined in silicone glassware Did you encounter this difficulty?

Barker At what temperature did you perform your tests?

Brinkhous At 28 degrees

Barker We had trouble in determining end points when the tests were done at room temperatures, but when we did the tests at 37 C a good solid clot formed and adhered to the silicone surface although not as tightly as a clot adheres to a glass surface

We did all the silicone coagulation time tests at 37 in a water bath. The glass coagulation time tests were done at room temperatures

Conley We have been very much interested in the effect of surface contract on blood from which the platelets have been

round a nucleated red cell from a tadpole We had seen (1929-30) similar appearances (Slide (*b*) V, 9) in rabbit and guinea pig erythroblasts from the heart blood of very young embryos

R F Furchgott [*Cold Spring Harbor Sympos Quant Biol*, *8 224 (1940)*] made a very fine study of essentially the same phenomenon which he calls stromatolytic forms" but he used ordinary hypotonic hemolysis to obtain the erythrocyte stroma as the starting element for his observations He found lithium perchlorate and potassium thiocyanate solutions especially good for inducing the appearances *I show this slide from a figure in his publication* As in our previous work on the platelets with which he was apparently not familiar Furchgott considers these phenomena essentially to be "myelin figure' formations Filaments from whole red blood cells were described by Kite (1914) and Auer (1933) This slide is reproduced from J Auer s paper [*Am J Med Sci* 186 *776 (1933)*]

Regarding the origin of the mammalian platelet from the megakaryocyte of the bone marrow (J H Wright, 1902) and perhaps elsewhere especially lungs [W H Howell and D D Donahue, *J Exp Med* 65, *177 (1937)*] I don t think any of us doubt the evidence I was especially impressed with some of the beautiful slides Dr Howell prepared and showed to me at the time of the cited publication I did, however have a recent interesting visit from Dr F Levy (Huntington W Va) to discuss this problem and his rather unconvincing revival of the idea that platelets are mere artefacts [*Amer J Clin Path* 15 *154 (1945)*] We can dismiss this last but I should like to review briefly some highlights of the megakaryocyte problem First let us observe the distinction made by Howell in 1895 between the polykaryocyte and the megakaryocyte

I have shown you an unpublished dark field photomicrograph of a typical polykaryocyte or osteoclast' from rabbit bone marrow kept alive in autologous serum at 38° C It is obviously a living dynamic cell slowly moving by typical ameba like pseudopods Its multiple nuclei are discrete and well differentiated Levy s confirmatory observations prove such a giant cell is formed by the fusion of histiocytic cells and it retains active phagocytic powers A very similar cell is the typical Langhan s giant cell of tuberculosis

Levy's studies clearly indicate that the megakaryocyte on the other hand is formed from a single histioid cell by an abnormal

formed at all in silicone treated tubes although they have formed in glass tubes

There is no thrombin in this plasma when it is introduced in the glass tubes As a matter of fact no thrombin is demonstrable until just a few seconds before clotting occurs as can be proved by oxalating the plasma serially as you go along The amount of prothrombin which is converted to thrombin during the coagulation process is so minimal that it is impossible to detect any loss of the prothrombin activity in the so-called serum although the amount of thrombin which is formed is adequate in most instances to convert all of the fibrinogen to fibrin

The behavior of normal plasma is in sharp contradistinction to what occurs in hemophilic plasma under the same conditions We have done this experiment on ten hemophilic individuals probably on about 25 different occasions and invariably the platelet free plasma is completely spontaneously incoagulable in glass tubes You can pour it shake it up in glass tubes and it won t clot although it clots very promptly on the addition of rabbit brain thromboplastin This is not a specific test for hemophilia because following either intravenous injection of heparin or in vitro addition of heparin to blood in a concentration which is too small even to affect the clotting time the same sort of thing occurs The plasma will not clot under these circumstances We recently showed that the activity of heparin is tremendously affected by the concentration of platelets When there are no platelets the heparin is extremely anticoagulant We are forced to conclude that there is in plasma a soluble substance which I consider to be a thromboplastin precursor which in contact with glass or other similar surfaces is changed to active thromboplastin We cannot deny the possibility that it may be derived from the platelets If it is however it is in *inactive* form at the time we put this plasma in the glass tubes There is no active thromboplastin or thrombin there and it is only after standing in glass tubes that clotting eventually occurs

removed I would like to tell you some of the data we accumulated on that subject This is a problem which has interested us now for a couple of years but because of the technical difficulties associated with the preparation of platelet-free plasma we have been very shy about making any comment about the results

We have done the experiment in over 50 normal individuals and in a great many people who were not normal Since our results were consistent, I think it is worth calling to your attention We have drawn blood from normal human subjects using silicone treated needles and syringes After introducing the needle into the vein we have withdrawn perhaps five cc of blood into the syringe and then very carefully changed syringes having washed out the needle with the first syringe and put on a dry clean syringe to withdraw the blood which we were going to use for a test That blood is immediately placed in a silicone treated tube in an ice bath and as quickly as possible within a few minutes placed in the high speed centrifuge in the refrigerated compartment and centrifuged at about 2 at 20 000 g for variable periods of times Sometimes a first centrifugation at 7 000 r p m for 15 minutes is done The supernatant plasma is removed and is recentrifuged in silicone treated tubes at 20 000 g In our experience it is sometimes no longer possible to demonstrate platelets after 10 minutes of centrifugation Undiluted plasma is entered directly in the counting chamber for platelet counts We have not observed any difference in the counts when the plasma is centrifuged for ten minutes or for very much longer periods of time Invariably when we remove this plasma from the silicone treated tube and place it in glass tubes at 37 it clots even in the complete absence of demonstrable platelets The clotting time is considerably longer than that of the blood from which the plasma was obtained We have shown the clotting time in glass tubes at 37 of the whole blood of two individuals, the clotting time of the platelet free plasma from those same individuals in glass tubes If other portions of the same plasma are simultaneously introduced in silicone treated tubes at 37 the clotting time is greatly prolonged In this instance it was 183 minutes in silicone tubes as opposed to 48 in glass In the other instance no clot ever formed in 48 hours in silicone tubes at 37 I say we have done this 50 times or more, and the results are invariable The clotting time in silicone treated tubes in often tremendously prolonged as compared to the clotting time in glass tubes In at least half a dozen cases no clots

formed at all in silicone treated tubes although they have formed in glass tubes

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ZETA POTENTIAL STUDIES

IRVING S WRIGHT and FRANK HORAN

Department of Medicine Cornell University Medical College

I think this is an appropriate time to present briefly some work that has been going on in our laboratory which is related to the work of both Dr Ferry and Dr Barker in that it pertains in a sense to electrical charges and also to surface problems

For some years we have been considering the possible relationship between electrical charges and the clotting mechanism This was very briefly referred to in theory by Dr Barker It is noted that glass contains strongly polar bonds between constituent atoms while plastics and paraffin have non polar bonds Polar bonds give rise to strong electrical forces in the neighborhood of the glass surface, one manifestation of which is the so called zeta potential The zeta potential arises from the electrical charges which accumulate on the glass surfaces and these charges usually negative, create a negative potential on the surface which may be as great as 200 millivolts, considering the potential in the middle of the liquid to be zero It is likely that different speeds of clotting may possibly be related in some degree to differences of electrical potential at different surfaces

Work has been carried on regarding this phenomena in our laboratories by Dr Frank Horan our physical chemist We have been working on the determination of changes in the zeta potential by the streaming potential technique in which capillary tubes are connected to containers at either end and under known pressure of nitrogen gas fluid is run through (Figs 14 and 15) We have been using a standard solution of potassium chloride and most of the experiments have been done with tubing made of vitreous silica Certain observations seem to be of considerable interest at least for discussion rather than in terms of final conclusions

The addition of very small amounts of fibrinogen to this standard potassium chloride solution reduces the zeta potential as determined by this method 95 99 per cent. This has been repeated on a number of occasions In other words if the zeta potential for the potassium chloride solution is 50 millivolts it is reduced to less than 1 millivolts even though the amount of fibrinogen added is

small such as 5 mg per 100 cc more or less If you add thrombin to the standard solution of potassium chloride it will lower the zeta potential about 75 per cent, that is to say fibrinogen will reduce the potential 99%—thrombin 75% We have also used heparin and paritol which is the synthetic anticoagulant which we are going to talk about tomorrow—both effective anticoagulants However instead of the zeta potential being reduced it is elevated slightly In other words instead of being down 99 per cent it might be up about 20 per cent The more interesting findings therefore indicate that these substances fibrinogen and thrombin which participate in the process of thrombosing produce this very marked effect We expect to continue these studies with varying pH concentrations effective inhibitors and types of glassware, etc

It seemed worth while to present this for your consideration It is conceivable that more attention should be paid to the importance of attraction and repulsion factors as they are related to the wall of the vessel in which the solution happens to be and also to these factors as they operate between particles in solution including blood

DISCUSSION

Ferguson Have you used heparin with thrombin?

Wright No that is an experiment on our list

Edsall Have you studied other proteins not involved in the clotting process like serum albumin?

Wright No it has taken us two years to build the proper equipment and Dr Horan has just made these preliminary observations Much more has to be done It is a method of approach which perhaps has not been considered as seriously as it might be It may or may not have ultimate value We were interested to find out whether anyone else in this group has had experience or has any information that might throw light on this method of attack Probably Dr Edsall has

Edsall No we have not worked on that

Wright We thought there might be some effect but to have these two substances which are involved in the thrombosing

mechanism produce such unequivocal and very profound effects really surprised us very much indeed

Edsall The salt concentrations were the same in the solutions with and without protein?

Wright The fibrinogen was dialyzed prior to its use, and this was a potassium chloride solution We tried to control that factor

Edsall Potassium chloride without fibinogen was used as a control?

Wright Yes

Fremont Smith When you measured the potential, you mea sured it from the inside of the tube to the outside while the potas sium chloride was in it?

Wright The original potential is this electrical charge here (illustrating) when you start the current going you measure the streaming potential which is the difference between the potential here and as it streams away the difference in this streaming potential dropped down from say 200 to 10 after adding fibrinogen or thrombin whereas normally it would stay up and conversely, the addition of heparin produced a slight elevation in readings

Tocantins Once you have passed through a solution like thrombin suppose the system is washed again with potassium chloride and another test run through Would that change the surface of the glass enough to affect the streaming potential?

Wright We took that into consideration The whole system was cleaned extremely carefully using acid etc and the potassium chloride was run through The results were the same as the original

Ferry Suppose you did not clean it thoroughly but ran some through without cleaning?

Wright With the idea of leaving a little residual thrombin in?

Ferry Yes

Wright All I can say is that minute amounts of fibrinogen or thrombin produced the change I mentioned 5 mg per 100 cc I want to point out that we do not consider any of this work is on a quantitative basis at present It is only on a relative one

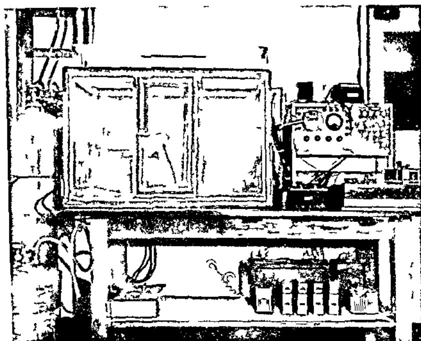


Fig 14 THE STREAMING POTENTIAL ASSEMBLY

Apparatus used for determination of zeta potentials of blood components

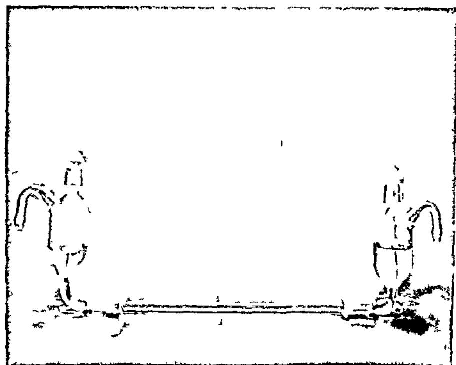


Fig 15 A CELL USED IN CAPILLARY STREAMING

These are relative figures The next experiment might be 140, and go down to 5 instead of 200 to 10 It was the relative drop that we were really surprised at The question which we were driving at ultimately was what happens in blood vessels under various changes of electrical potential and whether that can ever be measured Certainly that will be a long way from this experiment

Edsall You will try the silicone-coated tubes?

Wright As a matter of fact, we have already done some work with paraffin and silicone-coated material but the results are not far enough along to even discuss them here

Barker Gortner and Briggs did some work in 1923 with streaming electric potentials which led them to believe that platelets were attracted to a glass surface because the platelets were positively charged and the glass surface was negatively charged

Wright From time to time there have been a few sporadic observations—none in this country so far as we can find out Some German workers have done a little work and Pickering made a few observations This work is part of a cooperative project under an ONR contract with the College of Electrical Engineering at Cornell Professor Ballard of that school has become so interested that he is working intensively on the development of certain amplifiers which we hope will help to improve our equipment Professor Lloyd Woods from the Chemistry Department of Cornell is working with us on this project too

Quick It seems to me that it is much more logical to view this on the basis of electrical charges than on non wettable surfaces because the real surface that we are interested in is the endothelial lining of the blood vessels and as far as I know that is a wettable surface Later on if this work is true one must consider the electrical charges between the endothelial lining and the platelet since it is my impression that the platelets more or less bounce off the healthy endothelial lining There is an other factor that should be mentioned—a physical one in circulating blood the platelet because it is a very light body stays in the center of the stream and does not come in contact with the endothelial lining It should also be remembered that while healthy endothelium lining repels platelets injured endothelium does not Even a minor alteration as can be brought about by anoxemia may be sufficient to cause it to lose its ability to repel platelets

Wright It is possible—this is speculation of course—that it loses its negative charges for a number of reasons some of which may be increases in fibrinogen or thrombin, as illustrated here, which makes clotting a greater possibility, and, contrary wise, it is conceivable that larger amounts of antithrombins heparin or otherwise may increase negative charges and produce more repulsion This is easy to theorize on but it may be hard to prove, and we are the first to admit that

Jaques I might make some observations on silicone First, I want to thank Dr Barker for his kind remarks about our contribution and I would like to mention in this connection that the original idea of the use of silicone for the purpose of controlling blood coagulation came from Dr E T Felsted At that time Dr Felsted was a battalion medical officer at Camp Borden, Ontario He conceived the idea from news releases on the use of silicone as applied to various industrial and war uses for non wetting properties It was at his suggestion that the work was initially started at Toronto

There are a number of points on technique with regard to silicone that I think might be recorded at this time A minor point was that our original description advocated washing the vessels with dilute ammonia after the application of silicone That is a practice we dropped very early I do not believe there is any particular advantage in it

One problem I think that many have encountered is the question of the effectiveness of the coating We recommended applying the dri film at least three times to the fresh glassware before using it The purpose of this multiple coating is that in applying a coating of any material I believe it is general industrial experience that a first coating never gives a complete covering The difficulty with this repeated covering with dri film is that a very thick coating results It probably would be an improvement—and this has been suggested by Rochow and is being used by Maloney in Boston—to dilute the dri film with some hydrocarbon such as perhaps petroleum ether That is the one which Maloney is using to get a thinner coating Of course after applying the dri film the petroleum ether quickly evaporates the surface is washed with water as in the method originally described

Another technique which came out of the conference at Boston two weeks ago in which the use of plastics in preserving blood

was discussed was the question of the removal of the silicone coating Dr Rochow informs me that the method we recommended in the past chromic acid cleaning solution was about the worst thing we could recommend because it fixes the silicone to the glassware To remove the silicone from glassware all one has to do is prepare an alcoholic sodium hydroxide solution and soak the glass ware in this solution for an hour take it out rinse it off and the silicone has completely passed into the alcoholic soda solution

Then another point regarding the use of silicone which perhaps we did not indicate sufficiently in our first communication was that for really critical use such as when we want to obtain blood samples which do not clot at room temperature in silicone in three or four hours there are a number of points of technique which are due to unknown uncontrollable factors Dr Fidlar and I did a series of experiments on the effect of platelet counts and found on some days that the blood in the silicone tubes clotted before we could get it centrifuged while on other days our experiment was successful This problem becomes more acute the more critical the particular use of the silicone One of the factors that I think probably enters into the question is the blood air interface The drying of the blood at the blood air interface can certainly introduce errors in the determination of clotting time and it probably is true that once the blood is present in properly siliconized tubes with no admixture of thromboplastin the chief initiation of clotting comes from the interface between blood and air This may be due to drying, or to the low relative humidity or to the escape of carbon dioxide which with the exchange of gases across the red cell membrane must be causing changes in the plasma which probably promote clotting We have obtained silicone oils with the idea of using them the same way as paraffin oil for obtaining blood under oil with the hope that the silicone oil might be a little better than the paraffin oil

Finally of great importance I believe as everyone who has used silicone realizes is the problem of quantities of thromboplastin added to the blood as the blood is drawn Particularly important in this connection is the question of the needle The physical chemists say that it is very unlikely that any coating will coat the point of the needle so that one still has the steel cutting edge That is one factor There is also of course actual drawing up of thromboplastin into the syringe if care isn t taken as the needle passes through the vein A possible way out of this difficulty for

really critical uses of silicone which I would suggest, is the use of plastic tubing such as polyethylene tubing which can be introduced into a vein either in, or *on* a needle as Dr Tocantins uses it The polyethylene tubing is passed up the vein, clear of the puncture site and blood withdrawn through the tube, instead of the needle into the silicone vessels I would suggest this as a definite im provement in the technique certainly for critical uses of silicone Perhaps Dr Tocantins would like to add something to that

Tocantins The point to which Dr Jaques referred has to deal with the introduction of polyethylene tubing into the vein One way to catherize a vein using a polyethylene tube is to introduce a 15 gauge needle into the vein and pass the polyethylene tubing through the lumen of the needle up into the vein, then remove the needle leaving the tubing in the vein We have done that many times but the difficulty is that the caliber of the plastic tubing which one uses has to be very small It seemed to us that it might be better to do it the opposite way Instead of cannulating the needle with the plastic tubing, we cannulate the tube with the needle Thus the polyethylene tubing is used as a sleeve with the tubing over the needle After inserting the tube is squeezed while pulling the needle out That leaves the tubing in The "trochar' needle is 18 gauge and the caliber of the polyethylene tubing is about 16 gauge

That modification has worked well We have used it not for collection of blood but for longstanding heparinization when you simply leave the tube in the vein for several days to give blood transfusions The rate of flow is quite adequate

Jaques The point is the polyethylene tube gives us as much protection as the silicone surface If you wish you can treat the tube with silicone That is probably not very necessary but it gives protection not obtained by using a steel needle which cannot be completly coated with the silicone This probably is a very valuable addition to the use of silicone

Ferguson Instead of washing it with the ammonia you now just wash well with distilled water?

Jaques Yes sir

Barker Lampert reported that chrome steel surfaces had about the same effect on coagulation as plastic resin surfaces and that coagulation times of blood in chrome steel containers were con siderably longer than those in glass containers I wonder if draw ing blood into silicone coated tubes through uncoated chrome steel

needles would give shorter coagulation times than when blood is drawn through polyethylene tubing or silicone-coated needles

Jaques I agree that the chrome steel is nowhere near as great a stimulant to clotting as glass but I would suggest for very critical uses you probably need the extra insurance that you get with the polyethylene tubing over the chrome steel

Barker I suppose that once the tube is in the vein you can wait until the tissue thromboplastin is washed away from the puncture wound before drawing the sample

Quick I would like to ask whether there is any objection to using paraffin oil I have used silicone-coated tubes and paraffin oil as the covering to eliminate the air blood interface and found it rather successful

The other thing is I am surprised that Dr Seegers has not commented because in a paper which he published in *Blood* he presented a technique for getting blood whereby most of the possibilities of contaminating the specimen with tissue juice were eliminated By subjecting this blood to very high centrifugation he obtained samples which were completely incoagulable It seems that that is the answer at least for certain experimental work

Seegers I have no particular comment on that except what has already been said in print Our work has a disadvantage of course We went into the artery and ruined the animal You could hardly apply the technique we used to human beings We were very much interested in seeing whether we could get incoagulable blood and even then it was erratic with our refinements We many times got clotting but when we did there was only a small amount of fibrin which deposited It did not amount to very much and you could get successive crops We saw no difference between silicone tubes and non silicone tubes if you had centrifuged very thoroughly and if you had used really refined technique Perhaps it would be appropriate to comment on Dr Conley s findings which I presume were with human blood He was able to show that there was clotting despite the greatest care in technique He proposed I believe that this might be due to some kind of a circulating thromboplastin if I understood him correctly It still seems to me that there might have been liberation of thromboplastin at the junction where the syringe entered the needle or perhaps from some other source perhaps there was some damage to the platelets during centrifugation at low speed and when there was transfer to high speed centrifuging Even though he could not identify

any platelets by examination with a microscope, there might still have been platelet debris in there

While I certainly don t know the answer to Dr Conley s experiment I would hesitate very considerably to think that thromboplastin had not gotten into his materials I don t know how he could prove it one way or the other at the moment It seems to me it is a matter of opinion I lean toward the idea that probably it might have been contaminated due to a break in the technique somewhere

We were quite impressed with the work we did with dogs We worked very hard trying to get incoagulable blood and it is not easy when working with syringes

Conley Certainly as I said before, I cannot deny the possibility that we broke up some platelets in the performance of this experiment, thereby introducing thromboplastin into the blood However I think our experiments demonstrate convincingly that if any thromboplastin was so introduced it was inactive or was a thromboplastin precursor because coagulation in the silicone tubes was constantly prolonged as compared with the glass tubes This indicates that the glass tubes produce a change We know the silicone surfaces do not interfere with the activity of thromboplastin since the addition of dilute rabbit brain thromboplastin to the plasmas collected in siliconized and non siliconized glass gives the same clotting times

Wright May I ask whether you shake all the tubes, from the beginning of your test, or only begin the second tube after the first tube is clotted etc ?

Conley We have done it both ways The agitation of platelet-free plasma has much less effect than that of the platelet-rich, but it will accelerate clotting in the glass tubes

Quick May I make one remark concerning the clotting time test? It determines nothing more than the time required for sufficient fibrin to form to give the clot rigidity Even though the blood may be clotted much unchanged fibrinogen may still remain This is especially true in the case of hemophilic blood, for though the clotting time as determined by the Lee-White procedure may be one hour fibrin may continue to form for an additional hour or more

Knisely This is only related to the problem of getting blood out with a minimal mechanical breaking up of things

Flynn Dr Seegers made reference to a circulating thromboplastin I presume he was referring to the type of thing Chargaff reported You will recall that, in 1945 Chargaff [*Biol Chem* 160 *351 (1945)*] reported the effect of high speed centrifugation on the coagulation of fresh human plasma This plasma was oxalated placed in lusteroid cups and then subjected successively to 260 g for three minutes 1900 g for 20 minutes and 31 000 g for 150 minutes Samples removed at the end of each centrifugation period were recalcified and the clotting times determined It was noted that the clotting time increased as the plasma was centrifuged At the end of the high speed centrifugation the bottom of the tube contained a reddish brown pellet Furthermore Chargaff noted that this pellet brought about a very considerable shortening of the clotting time when added to the supernatant plasma In 1946 E Chargaff and R West [*J Biol Chem.* 166 *189 (1946)*] raised the question as to whether there is present in blood an additional factor exhibiting the centrifugal characteristics of the thromboplastic protein of tissue cells They studied the plasma of a human female who had a hemophilia like condition Centrifugal studies similar to those described above were done and it was concluded that the coagulation defect in the plasma of the patient was attributable in part to a marked deficiency in the blood of a clotting factor similar to the thromboplastic protein of tissue This coagulation defect could be overcome by the addition of purified thromboplastic protein of beef lung It seemed likely to them that a thromboplastic protein not unlike that isolated from tissue cells occurs in extravasated normal blood and contributes to its clotting properties

Dr Eugene T Standley and I repeated Chargaff s experiments on *human plasma* and in addition did the rather obvious thing of studying the sediments microscopically The actual technique was as follows

Sixteen (16) ml of carefully drawn human blood was added immediately to 2 0 ml of 0 1 M sodium oxalate This was centrifuged for three minutes at 260 g A small portion of the plasma from the top of the tube was drawn off for a clotting test A small amount was saved for a smear and microscopic examination A small amount near the buffy coat was also saved for a clotting test This plasma from the first centrifugation was known as *Sample A*

The remainder of Sample A was drawn off the cells and centrifuged for 20 minutes at 1900 g This was known as *Sample B*

A few ml were saved from the top and bottom of Sample B for clotting times It was noted that there was an abundant reddish white sediment covering the bottom of the tube A small portion of this was saved for microscopic examination, the remainder was *suspended in 1 0 ml of 0 9% saline This was called Suspended Sediment B*

The remainder of the plasma was centrifuged for 150 minutes at 31 000 g This was known then as *Sample C* A few ml of plasma were saved from the upper and lower portions of the supernatant plasma for clotting tests It was noted that a grey pellet of small size was present on the bottom of the tube A small amount was taken for microscopic examination The remainder was suspended in 0 5 ml of 0 9% saline This was called *Sediment C*

All centrifugations were carried out in cellulose nitrate cups

In the first experiment, 1 ml of each of the various samples of plasma was blown into a tube containing 0 1 ml of 0 9% sodium chloride and 0 1 ml of 0 02 M calcium nitrate with 0 42% sodium chloride Table 17 shows the results of these clotting tests The first figure of the clotting time represents the beginning of fibrin clot formation, the second solidification

TABLE 17

CLOTTING TIMES OF RECALCIFIED CENTRIFUGED PLASMAS

Plasma Sample	Centrifugation	Clotting Times
A supernate	260 g for 3 min.	175-200 sec
lower		165-201
B supernate	1900 g for 20 min	360 386 sec
lower		290 335
C supernate	31 000 g for 150 min	300 629 sec
lower		290-489

It can be seen that each centrifugation prolonged the clotting time of the plasma on recalcification These data are similar to those reported by Chargaff It is of interest to note that plasma taken from the lower portion of each tube after centrifugation

always with the exception of Sample A, clotted more rapidly than the supernatant portion

When supernatant plasma C was recalcified in the presence of Sediments B and C there was a reduction of the clotting time to the magnitude of that of the plasma centrifuged for 3 minutes at 260 g (see Table 18) Recalcification was carried out as before except that 0 1 ml of suspended sediment was substituted for the 0 1 ml of saline The formation of the fibrin clot was instantaneous and gelatinous in character in contrast to the slow formation ob served previously

TABLE 18

EFFECT OF ADDING SEDIMENTS A AND B TO SUPERNATE PLASMA C

Clotting Mixture		Clotting Times
Plasma C + Calcium + Saline	=	300-629 sec
Plasma C + Calcium + Sediment B	=	205 sec.
Plasma C + Calcium + Sediment C	=	207 sec

The platelet count of the supernatant portion of Sample A was within normal limits This slide* is a color microphotograph of the platelets The platelet counts on supernatant portions of Sample B and C could not be determined with the usual techniques since the number was *much diminished*

When the sediments were examined microscopically it was found that platelets were present in Sediments B and C The next two slides* are microphotographs of the sediments You will note that the platelets are clumped but otherwise have the same appear ance as the preceding slide There are fewer clumps of platelets in the sediment from C and from B The sediment from B contains red blood cells accounting for the reddish brown color of the pellet

In another experiment the same procedure was followed except that centrifugation at 1900 g was carried on for 240 minutes and the centrifugation at 31 000 g was carried on for 195 minutes

*Since these slides are in color they are not reproduced here

It was noted that samples corresponding to those in the first experiment had prolonged clotting times but restoration of the original clotting time occurred when the sediments were added back to the supernatant plasma of C Platelets were found again in both sediments

These experiments show that the pellet obtained by high speed centrifugation of human plasma is composed of platelets and, occasionally erythrocytes Perhaps there is a circulating thromboplastin, but Chargaff's results can be explained on the basis of the Brinkhous phenomena [*Am J Med Sci* 198 *509 (1939)*] concerning the effect of platelets on the conversion of prothrombin to thrombin Likewise of possible importance are the observations on platelet extracts by Mann *et all* [*Proc Soc Exp Biol and Med* 66, *83 (1947)*] and Ware *et al* [*Am J Physiol*, 154 *140 (1948)*]

Wright Are there any comments?

Seegers I would like to comment on Dr Flynn's and Dr Standley s observations It seems to me they have done a very fine thing I remember when the papers of Dr Chargaff first appeared I was quite puzzled by them Later at a conference on blood clotting in Washington someone praised Chargaff's efforts as extraordinary, which was embarrassing to me because I thought differently I am relieved that someone has repeated some of the work I am pleased that something more than prejudice has now come forth about this so called circulating thromboplastin

Tocantins I performed some of the experiments that Dr Flynn has reported stimulated just as Dr Flynn was by Dr Chargaff's observations My results correspond exactly with Dr Flynn's The bottom of the centrifuge tube when we centrifuged these plasmas for 48 hours in the cold in the angle centrifuge contained a little red pellet If one removes that, spreads it on a slide, and stains it there is no difficulty in finding platelets in it and a few red blood cells yet when the plasma was examined before it was put in the centrifuge for prolonged centrifugation, its platelet count was 2 000 per c mm One would consider it platelet poor plasma

Flynn I wonder if one can get a completely platelet free plasma

n we can learn something of the normal by e abnormal Hemophilia has aroused a great

deal of discussion Certainly there is something absent from the hemophiliac which is present in normal individuals If one wishes to consider it as a thromboplastin precursor one may do so I am not going to comment on that now but on another much more uncommon abnormal situation which I think sheds considerable light on the existence of an inactive thromboplastin in blood We have had the opportunity to study two patients with an unusual hemorrhagic diathesis Dr Quick has seen a similar patient and a small handful of other patients have been reported in the literature These patients had hemorrhagic diathesis with a remarkably prolonged coagulation time associated with the presence in the blood of a very potent circulating anticoagulant In one case this has been so potent that 5 parts of the patient s unoxalated native plasma when added to 1 000 parts of normal blood very markedly prolonged the coagulation time of normal blood While the clotting inhibitor is extremely potent the prothrombin time of these individuals is perfectly normal Furthermore if one adds to their plasma highly diluted thromboplastin the response is the same as one obtains with normal plasma so the anticoagulant is not antiprothrombic It seems that the action of this anticoagulant cannot be explained in any other way than that it prevents the conversion of inactive thromboplastin to active thromboplastin

Ferguson Let us consider the effect of surface on the colloids of the clotting system. If the surface action of the vessel containing the blood is important how much more important must be the surface action of the colloids of the blood itself particularly of colloids that are added extraneously to plasmas and to systems containing the isolated fractions that are participating in the clotting I would like to suggest the use of one word that we have mentioned on a number of occasions namely the term fibrinoplastic

A mixture of purified thrombin and fibrinogen coagulates just as readily in these non wettable siliconed tubes and in lusteroid tubes as in ordinary glass But there is a great difference in the speed of clotting if you add some colloid that presumably has an adsorptive effect on the reagents It has been known for a long time that many agents act thus for instance acacia as used in the Iowa two-stage method of assay of thrombin and prothrombin [*Am J Physiol* 137 *348 (1942)*] Protamine (salmine) is another example [*Am J Physiol* 130 *750 (1940)*]

It was noted that samples corresponding to those in the first experiment had prolonged clotting times but restoration of the original clotting time occurred when the sediments were added back to the supernatant plasma of C Platelets were found again in both sediments

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Flynn I wonder if one can get a completely platelet free plasma

Conley I think we can learn something of the normal by what we see in the abnormal Hemophilia has aroused a great

Ferry I presume that the sort of profibrin you have just described is entirely different from the sort of profibrin which is a partially polymerized fibrinogen

I might add to the list of substances which are fibrinoplastic polyvinyl alcohol and soluble starch both of which accelerate the clotting of purified fibrinogen by thrombin

Also I might mention one observation which is distantly related to the experiment described by Dr Wright, in which surfaces seem to play a part in the reaction between fibrinogen and thrombin We have noticed that at about pH 5 9 where the clot formation is very slow of the order of an hour that first a thin membrane of fibrin forms all around the periphery of the glass tube and also at the solution air interface It is easily detached by slight agitation forming a completely closed sac the inside of which is fluid later on the inside fills in I have supposed offhand that clotting took place at the surface first because of orientation of fibrinogen molecules near the interface which made it easier for the reaction to occur some of the unfavorable orientations being excluded

Wright Are there any other comments or questions?

Olwin Perhaps Dr Seegers hesitates to mention the fact that he and his group have discovered in platelets something which has a tendency to catalyze the thrombin fibrinogen reaction I wonder if we could have some discussion on that

Seegers I believe I did raise the question

Ferry At the time I had not thought of the non specific acceleration which Dr Ferguson has described which we have also seen in a couple of cases It could very well be that sort of thing

Seegers It is very interesting that the factor which can be derived from platelets has its effect even in presence of acacia In other words thrombin will clot fibrinogen at a certain rate and if acacia is added to the system the same amount of thrombin will clot it more rapidly If beside the acacia platelet extract is also added the fibrinogen clots even more rapidly

Jaques I raised the point that other factors might influence the clotting in siliconized glass and cause unexplained variation in spite of the fact that one may control or prevent the admixture of thromboplastin and also presumably may control the surface effect I wonder Dr Seegers if part of these unexplained variations may be due to changes in the clotting system within the vessel

P W Boyles and I recently investigated the effects of purified (calcium and oxalate-free) acacia on the thrombin fibrinogen reaction both as to clotting times and also the fibrin yields A typical experiment, in which the final percentage of acacia was varied up to 8 per cent is summarized in Fig 16

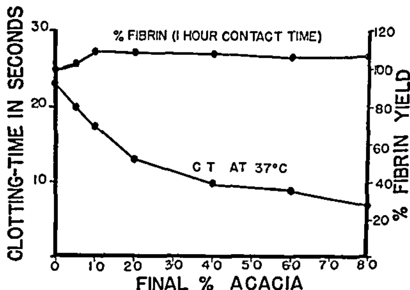


Fig 16

NOTE (1) The shorter clotting times as the acacia is increased with no evidence of any optimum
(2) No significant effects upon fibrin yield. With acacia the values were a little above 100 per cent but this could be experimental perhaps a slight opacity increasing the light absorption in the photoelectric colorimetry

The methods were noted previously in connection with the study of 'profibrin The similarity of results to those in connection with heated NaCl fibrinogen (Fig 11)* is noteworthy and leads us to conclude that *denaturation* merely provides a 'fibrinoplastic' effect and is unrelated to the questions of profibrin and the highly speculative European (Fischer Wohlisch Apitz) denaturase theory of the mechanism of action of thrombin

* Page 94

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such as conversion of the plasma Ac globulin to serum Ac globulin in the circulation and whether such conversion, occurring in vivo would mean that, to a considerable extent the value of the silicone technique is lost on the sample removed from the body In other words if I understand you correctly, you would propose that perhaps there could be circulating serum Ac globulin without our knowing it?

Seegers I am afraid that question has more consequences than is at first apparent We are inclined to believe that serum Ac globulin is equivalent and identical with Owren's Factor VI Owren has proposed that Factor VI is the activator of prothrombin It is obvious that a circulating serum Ac globulin would be incompatible with Owren s theory of the activation of Factor VI and of considerable consequence to his viewpoints I doubt that we have ever as yet encountered what might be truly called serum Ac globulin occurring physiologically I have often wondered whether or not one could detect physiologically the occurrence of serum Ac globulin I don t think the possibility is out of the question

If one were to test for the presence of serum Ac globulin I would predict that it would be found because in those instances where clotting was obtained with the best silicone technique serum Ac globulin in appreciable quantities was identified

It might be possible then putting aside the question of serum Ac globulin occurring physiologically, that after removal of the sample conversion of plasma Ac globulin to serum Ac globulin is due to factors other than surface factors controlled by the silicone such things, for example as the blood air interface disturbances of the surface of the red cell etc

I think it is a very difficult question you raise It involves some very careful technique

ON THE CONVERSION OF PROTHROMBIN TO THROMBIN

JOSEPH E FLYNN and EUGENE T STANDLEY

Department of Pathology Columbia University

Between 1938 and 1948 the work of a number of laboratories showed that a deficiency of certain factors causes prolongation of the conversion rate of prothrombin to thrombin These factors have been variously termed (a) convertibility factor [E D Warner K M Brinkhous and H P Smith *Proc Soc Exp Biol and Med* 40 *197 (1939) Am J Physiol* 125 *296 (1939)*] [W H Seegers K M Brinkhous H P Smith and E D Warner *J Biol Chem* 126 *91 (1938)*] [C A Owen G R Hoffman S E Ziffern and H P Smith *Proc Soc Exp Biol and Med* 41 *181 (1939)*] [H P Smith *Essays in Biology in honor of Herbert M Evans* page *549* University of California Press *(1943)*] (b) labile factor [A J Quick *Am J Physiol* 140 *212 (1943)*] (c) plasmakinin [K Laki *Studies from the Institute of Medical Chemistry University of Szeged* S Kargu Basel New York III *97 (1943) Schweiz Med Wchnschr* *74 13 (1944)*] (d) factor V [P A Owren *Lancet* 1 *446 448 (1947) The Coagulation of Blood* Oslo J Chr Gundersen 326 pp *(1947) Acta Med Scand Suppl* 124 *(1947)*] (e) prothrombin accelerator [P Fantl and M Nance *Nature* 158 *708 (1946)*] and (f) accelerator globulin [A G Ware M M Guest and W H Seegers *J Biol Chem* 169 *231 (1947) Science* 106 *41 (1947) Am J Physiol* 152 *567 (1948)*] [A G Ware and W H Seegers *J Biol Chem* 174 *565 (1948)*] We refer to these substances as accessory factors Our work indicates that all of the factors enumerated have essentially the same effect and are probably one and the same thing The conclusion concerning their equivalence is based on the following experiments

Fig 17 shows the effect of Owren s factor V on the conversion of purified prothrombin as determined by a two-stage method of prothrombin assay [E D Warner K M Brinkhous and H P Smith *Arch Path* 20 *163 (1935) Am J Physiol* 114 *667 (1936)*] [W H Seegers *Blood Clotting and Allied Problems* 1 *152 (1948)*] This experiment is similar to those described by A G Ware and W H Seegers [*Am J Physiol* 152 *567 (1948)*]

such as conversion of the plasma Ac globulin to serum Ac globulin in the circulation and whether such conversion occurring in vivo would mean that to a considerable extent the value of the silicone technique is lost on the sample removed from the body In other words, if I understand you correctly you would propose that perhaps there could be circulating serum Ac globulin without our knowing it?

Seegers I am afraid that question has more consequences than is at first apparent We are inclined to believe that serum Ac globulin is equivalent and identical with Owren s Factor VI Owren has proposed that Factor VI is the activator of prothrombin It is obvious that a circulating serum Ac globulin would be incompatible with Owren's theory of the activation of Factor VI and of considerable consequence to his viewpoints I doubt that we have ever as yet encountered what might be truly called serum Ac globulin occurring physiologically I have often wondered whether or not one could detect physiologically the occurrence of serum Ac globulin I don t think the possibility is out of the question

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It might be possible then, putting aside the question of serum Ac globulin occurring physiologically that after removal of the sample conversion of plasma Ac globulin to serum Ac globulin is due to factors other than surface factors controlled by the silicone such things for example as the blood air interface, disturbances of the surface of the red cell, etc

I think it is a very difficult question you raise It involves some very careful technique

using the accelerator globulin In the test purified bovine prothrombin* was incubated with calcium and purified thromboplastin The thromboplastin contained only a trace of factor V At intervals some of the material was removed and added to a standard fibrinogen The ordinate shows the clotting time from which the thrombin unitage can be calculated The abscissa shows the time of incubation The upper curve represents the conversion time when no factor V was added but obviously some factor V was present as an impurity otherwise none of the prothrombin would have been converted The time required for maximum conversion of prothrombin to thrombin was 35 minutes The lower curve shows the effect of adding Owren s factor V Here maximum conversion occurred in three minutes It will be noticed that under the conditions of the experiment the conversion time was not reduced below the usual level for bovine plasma In this type of experiment a three minute conversion seems to be the irreducible minimum for the bovine species

The question can be raised as to why the thrombin yield is lower with sub-optimal amounts of factor V than with optimal amounts This could be explained on the assumption that thrombin inactivates prothrombin An alternate hypothesis favored by Owren is that factor V is consumed in forming thrombin

Fig 18 shows a similar experiment except that the factor V is replaced by Laki s factor plasmakinin [K Laki *Studies from the Institute of Medical Chemistry University of Szeged* S Kargu Basel New York III *97 (1943)*] and [*Schweiz Med Wochenschrift* 74 *13 (1944)*] This factor likewise increased the conversion rate as well as the thrombin yield The activity of Laki s factor is less than that of Owren s factor V It will be recalled that the preparation of plasmakinin is an extremely simple one consisting of precipitation of plasma by 25 per cent saturation with ammonium sulphate After centrifugation the precipitate was dissolved and dialyzed against distilled water The fibrinogen was removed from the dialysate by adding a minute amount of thrombin The plasmakinin we prepared contained 75 units of prothrombin per ml as an impurity In the amounts used in the experiments of Fig 18 the plasmakinin contributed 0 015 units of prothrombin to the final clotting mixture—obviously an amount too small to be significant

* 5 000 units per mg of nitrogen

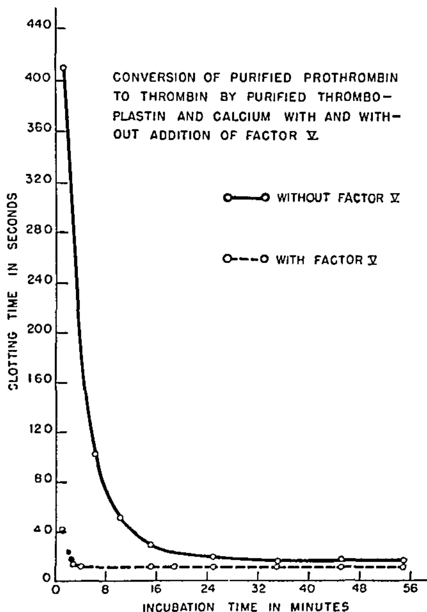

CONVERSION OF PURIFIED PROTHROMBIN TO THROMBIN BY PURIFIED THROMBO- PLASTIN AND CALCIUM WITH AND WITH- OUT ADDITION OF FACTOR V.
WITHOUT FACTOR V
WITH FACTOR V
CLOTTING TIME IN SECONDS
INCUBATION TIME IN MINUTES
440
400
360
320
280
240
200
160
120
80
40
0
8
16
24
32
40
48
56

FIG 17

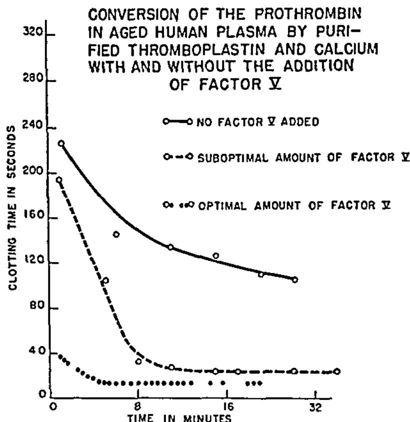


FIG 19

curve shows the conversion time and thrombin yield when no factor V was added Maximum conversion occurred in 30 minutes and the thrombin yield was less than one fourth unit The middle curve exhibits the effect of a sub-optimal amount of factor V Here the time of maximum conversion was 15 minutes and the thrombin yield was 0 5 units In the lowest curve an excess of factor V was used The time required for maximum conversion was 5 minutes and thrombin yield 1 unit This conversion time is normal for human plasma with the two-stage method of prothrombin assay We have never been able to lower the conversion of human plasma at the one unit level below this value whereas values as low as 1 minute are readily obtained with canine plasma

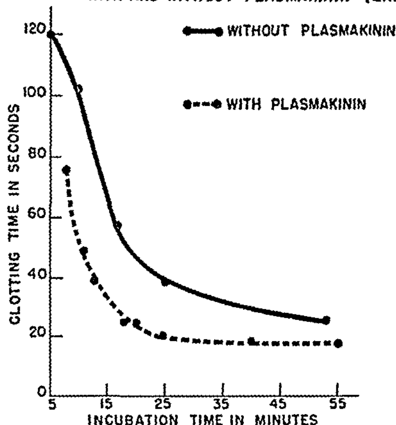


FIG 18

We then repeated this experiment, using stored blood [A J Quick, *Am J Physiol* 140 *212, (1943)*] [A J Quick and M Stefanini, *J Lab and Clin Med 33 819 (1948)*] and we concluded that it lacks factor V Fig 19 shows the effect of Owren s factor on the prothrombin unitage of stored blood This experiment is another replica of those published by A G Ware and W H Seegers, [*Am J Physiol* 152 *567 (1948)*] except that here the purified prothrombin is replaced by aged, oxalated human plasma In the chart, the ordinate gives the clotting time in seconds The abscissa represents the incubation time The upper

the two-stage assay When plasma is the test substance we proceed first of all to convert the plasma into serum by a special technique to be described later

The subsequent procedure consists of adding a constant amount of prothrombin (2 5 units) to the incubating mixture and then determining how much test substance must be added to convert 50 per cent of the prothrombin to thrombin within a three to five minute reaction period The necessity of fixing the amount of prothrombin added to the incubating system is apparent from Table 19 This table shows there is a reciprocal relationship between the amount of factor V needed to produce 1 25 units of thrombin and the amount of prothrombin present in the incubating mixture For purposes of comparison the factor V activity can be reported in terms of its dilution factor as dilution units or in percentage by making some source of factor V a standard reference frame

Reference has been made to the use of a special technique for assaying plasma When recalcified human oxalated plasma or human serum obtained by permitting the spontaneous coagulation of blood is used as the test substance we found that in either case the serum contained 90 100 dilution units of factor V However if serum is prepared by permitting whole blood to clot in the presence of calcium* it contains 300 350 dilution units of factor V Furthermore the factor V content in the latter instance is stable for several hours permitting one to wait until all the prothrombin is converted and the thrombin inactivated by anti thrombin

Just why calcium has a protective action on the factor V content of human serum is not known The phenomena is undoubtedly analagous to Quick s observation [A J Quick *Proc Soc Exp Biol and Med* 62 *249 (1946)*] that the presence of calcium preserved the labile factor content of hemophilic blood The loss of factor V in serum from spontaneous coagulation of human blood is not related *per se* to fibrin or thrombin since both of these substances are present when whole blood clots in presence of excess calcium The calcium alone is not responsible since a comparable increase in the calcium content of the incubating mixture (with no factor V) produces no effect on the prothrombin unitage of stored plasma

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3 0 ml of isotonic calcium chloride added to 3 0 ml of whole blood

Fig 20 shows the effect of adding varying amounts of factor V to the thromboplastin when stored blood is assayed by the two-stage method The abscissa indicates the amount of factor V added the ordinate gives the prothrombin unitage as determined from the dilution factor of the plasma in the final clotting mixture When no factor V was added no measurable thrombin was obtained When the final clotting mixture contained a concentration of 0 004 ml of a standard preparation of factor V, the titer rose to the 300 unit level It is evident that 0 004 ml of factor V was needed to secure maximal conversion With small amounts, the final clotting mixture contains both thrombin and unconverted prothrombin

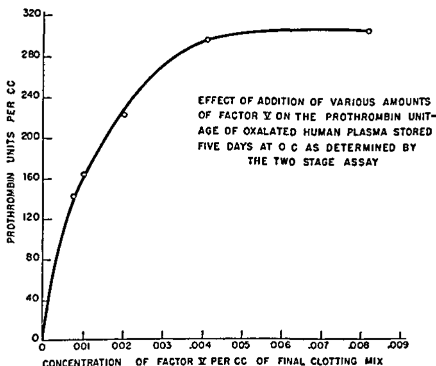


Fig 20

Somewhat aside from our present discussion, we make it a practice to use stored plasma for the assay of factor V This is done by adding small increments of a test substance either directly to the defibrinated stored plasma or to the thromboplastin used in

To return again to the main discussion Table 20 shows the effect of Laki s factor [K Laki *Studies from the Institute of Medical Chemistry University of S eged* S Kargu Basel New York III *97 (1943) Schweiz Med Wchnschr 74 13 (1944)*] on the prothrombin unitage of stored plasma With no plasmakinin added to the thromboplastin no prothrombin units were obtained With the addition of plasmakinin 150 units were obtained It will be noted that the effect is similar to that obtained with factor V In calculating the units obtained a deduction was made for the amount of prothrombin present in plasmakinin The amount of prothrombin obtained increased as the concentration of plasmakinin increased

TABLE 20

EFFECT OF VARYING AMOUNTS OF PLASMAKININ ON THE TWO STAGE ASSAY OF STORED OXALATED HUMAN PLASMA

Concentration of plasmakinin per ml of final clotting mixture	Prothrombin Units per ml of stored plasma
0	0
0 025	100
0 05	151

These various experiments on purified prothrombin and aged plasma establish the equivalence of the labile factor factor V of Owren and plasmakinin Subsequent experiments of this type also indicate identity with the accelerator globulin

An effort was then made to devise a one-stage technique which could be employed for an additional comparison of these various preparations It was hoped that the one stage prothrombin test of Quick could be modified and rendered suitable for this purpose The standard preparations of calcium and brain extract were em ployed Stored human plasma deficient in the labile factor was used in place of normal plasma The system thus had little or no tendency to clot By adding test material in varying amounts it would seem that the restoration of clotting power might provide a quantitative measure of accessory substances such as the labile

TABLE 19

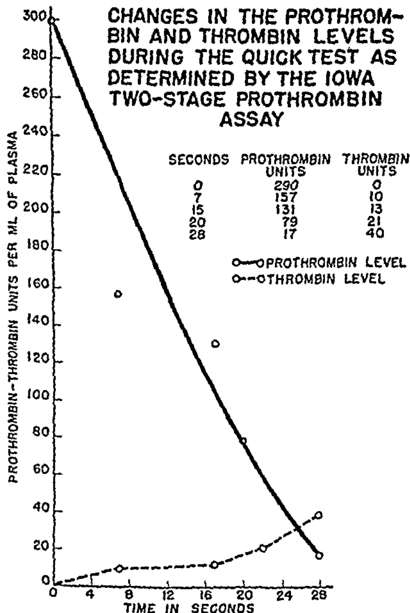
AMOUNT OF FACTOR V NEEDED TO FORM 1 25 UNITS OF THROMBIN WITHIN FIVE MINUTES FROM VARIOUS AMOUNTS OF PROTHROMBIN

Observation	Units of prothrombin (from stored plasma) added to incubating mix	Ml of standardized factor V preparation per ml of incubation mix	Units of thrombin obtained in a five minute reaction period
1	1 25	62 5 $\times 10^{-5}$	1 25
2	2 50	36 25 $\times 10^{-5}$	1 25
3	5 00	13 8 $\times 10^{-5}$	1 25
4	7 50	11 4 $\times 10^{-5}$	1 25
5	10 00	9 4 $\times 10^{-5}$	1 25
6	12 50	8 9 $\times 10^{-5}$	1 25

In all determinations the two stage method of assay was used The incubating mixture contained excess thromboplastin (lung extract) buffer acacia and optimal calcium The prothrombin source was stored oxalated human plasma (aged 6 days at 0 C) deficient in factor V The prothrombin unitage was determined by first assaying stored plasma with an added excess amount of factor V The plasma was suitably diluted so that the incubating mixture contained only 1 25 units of thrombin For subsequent determinations the amount of prothrombin in the incubating mixture was varied by using different dilutions of the stored plasma After 1 3 5 minutes of incubation 0 4 cc of the incubating mixture was added to 1 0 cc of fibrinogen and clotting time noted

The end point was the amount of factor V needed to give a 15 second clot at *the end of five minutes Such a clotting time represents 1 unit of thrombin* in the final clotting mixture or 5/4 of a unit (1 25) in the incubating mixture It is obvious that the 15 second clot is obtained by trial and error The above data does not show the results of trial and error but merely the amount of factor V needed to produce 1 25 units of thrombin The factor V used was prepared after the technique of P Owren [*Coagulation of Blood* J Chr Gundersen Oslo 1947 also *Acta Med Scand Suppl* 124 *(1947)*]

For routine assays of factor V, the incubating mixture contains 2 50 units of prothrombin By trial and error enough factor V is added to give exactly 50% conversion of the prothrombin to thrombin within five minutes The 50% conversion figure was selected on the basis that in this range small increments of factor V produce noticeable changes in the amount of prothrombin converted hence the sensitivity is greater than if 100% conversion were selected The use of 2 50 units of prothrombin requires a high dilution of stored plasma thereby minimizing the effect of antithrombin The assay can also be done with purified prothrombin instead of stored plasma



CHANGES IN THE PROTHROMBIN AND THROMBIN LEVELS DURING THE QUICK TEST AS DETERMINED BY THE IOWA TWO-STAGE PROTHROMBIN ASSAY
SECONDS
PROTHROMBIN UNITS
THROMBIN UNITS
0 290 0
7 157 10
15 131 13
20 79 21
28 17 40
PROTHROMBIN LEVEL
THROMBIN LEVEL
PROTHROMBIN-THROMBIN UNITS PER ML OF PLASMA
TIME IN SECONDS
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
4
8
12
16
20
24
28

FIG 21

factor, factor V, plasmakinin, or Ac globulin To a certain degree these hopes seemed at first to be realized It was difficult, however, when dealing with preparations which contained prothrombin to control the amount of prothrombin initially present There were also difficulties in securing constancy in the reactivity of the fibrinogen of the mixture In addition, we encountered variations due to factors which still remain unanalyzed This experience led us into a study of the basic principles of the one stage procedure itself

Fig 21 shows typical results of our studies With normal human plasma, rabbit brain and calcium a clotting time of 15 seconds was obtained An effort was then made to study the rate of thrombin formation during this 15 second interval To do this the test was repeated many times with identical reagents Samples were removed at selected intervals in order to follow the changing levels of prothrombin and thrombin The thrombin curve, shown at the bottom of the chart was obtained by transferring small portions, at selected intervals, into oxalated saline The oxalate checked the process of conversion more or less completely A portion was then mixed as rapidly as possible with an appropriate amount of fibrinogen The clotting time, together with the dilution factors made it possible to calculate the amount of thrombin formed at the moment selected for study The curve shows that the thrombin titer had reached the 10 unit level at the end of 8 seconds of incubation As stated already clotting occurred at the end of 15 seconds It was of interest nevertheless to follow the thrombin titer beyond this point In such experiments, the fibrin clot was removed and the titer of the residual fluid was determined It is seen that the titer rises to the 40 unit level at the end of 28 seconds

The upper curve shows the prothrombin titer throughout this same interval In this case too the samples removed were treated with oxalated saline and then immediately subjected to the two stage technique of assay The titer of course represented the summation of prothrombin and thrombin but in drawing the upper curve the thrombin titer was deducted from the total the upper curve thus represents merely the prothrombin present It will be seen that 300 units per ml were originally present in the reaction mixture The titer fell off rapidly and very little prothrombin remained at the end of 28 seconds It is of interest to note what had happened at the mid point in the curves, immediately prior to clotting of the one-stage test Thus at the end of 14 seconds, the

direct type J W Trevan and R G Macfarlane (*Annual Report of Med Research Council* for the year 1936 1937 p 143) noted that the hemostatic action of Russell s viper venom was greatly accelerated by adding tissue extract or lecithin The effect was confirmed by L J Witts and F C G Hobson (*Brit Med J* 575 May 9 1942) and (*Brit Med J* 862 Dec 21 1940) by J B Leathes and J Mellanby [*J Physiol* 96 *39 (1939)*] by R C Page E J deBeer and M L Orr [*J Lab Clin Med* 27 *830 (1942)*] by A J Quick [*Am J Clin Path* 15 *560 (1945)*] and by A. Crosbie (*Brit Med J* 268 Feb 22 1941) These reports dealt with one-stage techniques

We repeated and confirmed these experiments using an alcohol extract of asolectin instead of lecithin Thus Table 21 shows the clotting times when various mixtures were substituted for the thromboplastin used in the Quick one stage prothrombin assay When asolectin was substituted for thromboplastin the clotting time was 180 seconds With viper venom alone the clotting time was 22 9 seconds with dessicated rabbit brain it was 12 0 seconds With viper venom and asolectin it was 10 2 seconds With viper venom + asolectin + dessicated rabbit brain the clotting time was 6 9 seconds Quick has published similar data [*Am J Clin Path* 15 *560 (1945)*] In all determinations the same amount of oxalated human plasma was used and the final volume was the same for all determinations The asolectin was obtained from Associated Concentrates Inc Their analysis shows that the asolectin is composed of about 30 per cent lecithin 30 per cent cephalin 35 per cent lipositol and the rest is soya oil

TABLE 21

CLOTTING TIME USING VARIOUS THROMBOPLASTIN MIXTURES IN QUICK S ONE-STAGE PROTHROMBIN ASSAY

Type of Thromboplastin	Clotting Time
Asolectin	180 0 seconds
Viper Venom	22 9 seconds
Dessicated Rabbit Brain	12 0 seconds
Viper Venom + Asolectin	10.2 seconds
Viper Venom + Asolectin + Dessicated Rabbit Brain	6 9 seconds

thrombin titer was nearly 20 units whereas the prothrombin titer had fallen to the 120 unit level It is thus evident that approximately *180 units of prothrombin had disappeared during these 14* seconds It seems certain that this prothrombin must have given rise to 180 units of thrombin nevertheless only 20 units of thrombin remained It would appear that approximately three fourths of the thrombin had disappeared before clotting occurred and this is no doubt due to the action of antithrombin If this is true, it would appear that the one-stage test measured merely a minor fraction of the thrombin formed—a vanishing fraction at that The reliability of the test must depend in great degree upon constancy of this complicating factor Even a small shift in the rate of inactivation can be expected to produce a large alteration in the amount of thrombin present This undermines the entire philosophy of the one stage test

Also it should be pointed out that anything which alters the speed of conversion will upset this delicate balance of variables When there is a deficiency in factor V there is slow conversion of prothrombin into thrombin

There is thus a longer time interval for the action of thrombin on fibrinogen At the same time, there is more opportunity for the inactivation of thrombin by antithrombin Who can say that *these two complications will offset each other exactly or to an* equal degree from one case to another? It is evident that the one stage test depends not merely upon the amount of prothrombin present, but also upon the shape and intensity of the conversion curve, and upon the amount and activity of antithrombin present It is indeed certain that some of these complicating variables may at times dominate the picture Our own studies show that variations of unaccountable nature do occur In canine plasma for example the thrombin titer rises rapidly the prothrombin titer falls precipitously, and clotting occurs within the first 10 seconds Doctor Quick has been led to conclude that canine plasma is very rich in prothrombin We are convinced however that this is not the case The rapid clotting is due to rapid conversion aided perhaps by slow action of antithrombin We have not yet been able to ascertain the cause of the rapid conversion In our opinion, it is probably not simply the result of a superabundance of factor V It seems likely that other types of accelerator exist

This indirect evidence for the existence of clot promoting substances of a new type is supported by certain evidence of more

chart indicates the amount of asolectin added to the assay It will be noted that the titer rises to the level of about 140 units after which it falls This shows that some component of asolectin has at first a stimulating effect but with larger amounts it has an inhibiting effect *This inhibiting effect recalls the experiments of* Tocantins and Overman in which they found evidence for a thromboplastin inhibitor as evidenced by a decrease of thrombin yield *A similar* inhibiting effect was observed in a one stage method of analysis—namely if the concentration of asolectin was increased beyond a certain critical amount the clotting time became prolonged *By using low concentrations of asolectin* we diluted the inhibitor out of existence whereas Tocantins and Overman concentrated the inhibitor and eliminated the stimulator

Fig 23 shows a similar experiment with asolectin but using purified prothrombin instead of aged human plasma This experiment was also performed with small amounts of thrombin Here the thrombin titer is plotted against increasing amounts of factor V on a semi log scale The upper curve is presented with a supplement of asolectin the lower without a supplement With purified prothrombin the asolectin gives 20% to 30% greater yield than when not present In these experiments the asolectin was used in low concentrations If the concentration was increased beyond a certain level the inhibitor effect was again demonstrated We have found our purified prothrombins to be variable in response to the asolectin extract again suggesting that the active principle is present in some batches as a contaminant

We have also conducted experiments using purified lecithin *instead of asolectin Similar results were obtained We would not* stress this point because the lecithin was certainly not a chemically pure preparation We can only say that the active component appears to be a lipoid possibly lecithin

The question can be raised as to whether the active principle in asolectin and lecithin is really a new factor We believe it is since our experiments show that it supplements any combination of known factors This would not occur if the asolectin was just thromboplastin or factor V Although this factor must be purified before we can be certain as to its exact role in blood clotting we believe these experiments add additional weight to the original work of Trevan and Macfarlane concerning a new factor present in lecithin preparations

The two-stage prothrombin assay was also used to study this factor since this technique permits us to measure the total amount of thrombin formed Preliminary experiments with the two-stage technique showed that asolectin is not a substitute for factor V On the other hand the active principle of asolectin is probably present as a contaminant in some of our standard clotting reagents For this reason, its effect was best demonstrated when suboptimal amounts of certain reagents were used The technique was other wise the two stage procedure using calcium lung extract, stored human plasma that was largely free of factor V Preliminary experiments showed the addition of a large supplement of factor V would produce a titer of 300 Iowa units calculated as prothrombin or thrombin We used, however a suboptimal amount of factor V and as a result, a titer of only 69 units was obtained as is shown in Fig 22 You will recall that in the two-stage prothrombin assay the plasma is diluted so that incubating mixture contains approximately one unit of prothrombin The total unitage is calculated from the dilution factor of the plasma The figures shown on the ordinate represent the total unitage The abscissa of this

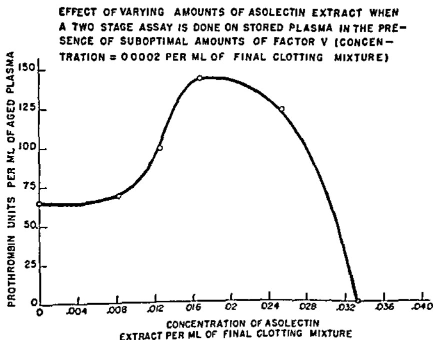


FIG 22

in serum obtained by permitting blood to clot in the presence of excess calcium the factor V content is higher in serum prepared by not adding calcium. Furthermore the factor V is far more stable when excess calcium is present We use protective merely for description and make no pretense as yet, to interpret the fundamental interplay of calcium with the labile factor

Wright Dr Seegers are you satisfied with that definition?

Seegers I am satisfied with that definition and I think I understand exactly what Dr Flynn means The situation is quite complicated We have covered it fairly well in three current papers in the American Journal of Physiology in which we studied the disappearance of factor V or what we call plasma Ac globulin in various species—how it disappears in serums and so on

We have also made a study of the factors which influence factor V or plasma Ac globulin in various storage plasmas and we have been able to show that oxalated bovine plasma contains factor V or plasma Ac globulin in full concentrations for many long months In the human the situation is somewhat different and I cannot possibly summarize the paper which we have written on that I think it is best simply to refer to it in the American Journal of Physiology It is quite concentrated

I believe it would be an excellent accomplishment to have a simple test for measuring quantitatively factor V activity and it seems to me that Dr Flynn has gone a long way in that direction I think he realizes as everyone else must realize that the criticisms which can be leveled at a one-stage method for prothrombin analysis or similar ones can in part be leveled at a one stage analysis for factor V Theoretically to study the action of factor V and factor VI or serum Ac globulin I think it is essential to use a two-stage analysis or even what might be called a three-stage analysis I am sure Dr Flynn realizes that it makes the situation very complicated Whereas the two stage analysis was very difficult to use for working out the theoretical aspects of prothrombin as compared with the one-stage analysis the two-stage analysis for Ac-globulin is all the more complicated We have had any amount of difficulty but I think it has been profitable to go into the theoretical aspects

Dr Owren believes that factor V is consumed in the reaction I recall that at one time we believed that thromboplastin is consumed in the reaction But in the case of thromboplastin we have

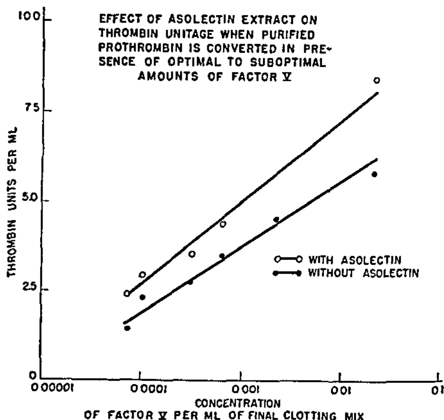


FIG 23

DISCUSSION

Seegers Dr Wright I would first of all like to express my appreciation of this excellent presentation I think by and large it speaks for itself and needs no comment I thought perhaps there might be some help from another viewpoint

The statement was made that calcium has a protective action on the accelerator, and I am inclined to the view that perhaps the action is not a protective one I do not know however, how the action of calcium can be explained

Wright Could we have a definition of protective action'? Perhaps we all do not think in terms of the same definition Let us be certain in any event

Flynn The term protective action" was one borrowed from Quick The demonstrable facts which lead us to its use are

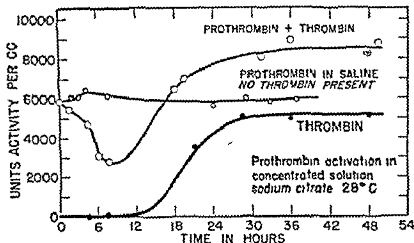


Fig 24

The figure shows three curves (1) the formation of thrombin when purified prothrombin is dissolved in a 30 per cent solution of sodium citrate (2) the concentration of prothrombin plus thrombin activity in the same solution of prothrombin in 30 per cent sodium citrate (3) the stability of the same prothrombin preparation when simply dissolved in saline solution

production is very rapid At the end of about thirty hours the maximum yield has been obtained with no further production of thrombin The curve looks very much like the curve for autocatalytic activation of prothrombin but so far we have not actually applied the necessary mathematical formula to be able to say that it is autocatalytic In the meantime one can place at room temperature a control prothrombin which will retain its full activity for the full period of time This control is also plotted in Figure 24

In measuring the prothrombin plus thrombin activity we found that coincident with the appearance of thrombin the prothrombin activity goes down I presume that is because thrombin is inactivating prothrombin and we are going through the phase which I discussed at the meeting last year Then coincident with the appearance of appreciable amounts of thrombin the prothrombin activity goes back up quickly returns to the point of beginning and many times overshoots the mark of the point of beginning I will not discuss this last fact further because that has occupied many long hours of our efforts and there is more to it than just this

been able to recover active thromboplastin after it seemed to have been consumed At the meeting here last year, I made some theoretical deductions as to why these factors appear to be con sumed in the reaction but actually are not We now have some new data on the activation of prothrombin which I believe will go a long distance towards classifying and clarifying what Dr Flynn has referred to as accessory factors

We have information which indicates that prothrombin con tains all the *necessary amino acid building stones needed for* the thrombin molecule As a preliminary to the experiment I am about to show, I would like to say that the basis on which this work is built is the use of purified prothrombin the best that we can make We employ a routine system of preparing the material once per day We make five lots per week It takes about 16 man hours to make about 50 mg of the material As a general rule we are able to get material of high potency in the neighborhood of about 20 to 25 000 units per mg of tyrosine Many times the activity is much less but never does the activity go far beyond 25 000 We think that fact is an additional criterion of purity beyond the physical chemical methods which have already given some indication as to where we stand in that respect

We are reasonably certain that it is free of thromboplastin *We feel absolutely certain that it is free of accelerator globulin* We were able to show that if one places such a prothrombin preparation in solution it will remain stable for a period of about 24 hours at room temperature If a very small amount of thrombin is added to such a prothrombin preparation the activity of the prothrombin will decrease and remain low If one adds rather large amounts of thrombin to the prothrombin preparation there is first a decrease in prothrombin activity, and then the prothrombin activity will regenerate with the formation of some free thrombin That was our clue We reasoned that perhaps one can activate purified prothrombin without any accessory factors and accordingly made a search *to find such activators The one which* we now use is sodium citrate in high concentration If one takes a prothrombin concentration of 10 000 units per cc in other words a high concentration and adds sodium citrate up to 30 per cent, the prothrombin will be activated at room temperature (Fig 24) A small amount of thrombin is present at about six hours After ten hours there is slightly more At about twelve hours there is an appreciable amount of thrombin then thrombin

talking about the prothrombin complex I might also recall that when I asked Dr Quick to explain yesterday what he meant by prothrombin complex he did not answer Our work does fit in line with what the Cornell group said yesterday about being able to activate prothrombin without having had calcium or other factors and at least from my viewpoint this all fits From a futuristic standpoint we are very happy about the discovery of sodium citrate for the reason that we have spent considerable effort and it is still difficult to get purified prothrombin We had difficulty in satisfying ourselves that we were right about having essentially a purified substance when certain physical chemical measurements did not seem to bear that out as well as they might have and we were in the difficult position of having to go from prothrombin to thrombin by adding Ac globulin and thromboplastin with all of their impurities only to end up with thrombin which must then be purified This now places us in the position of being able to go straight through and measure by physical-chemical means the prothrombin at this point going over to thrombin with sodium citrate and measuring what you have here Not only are we able to study those activities with respect to fibrinogen but also we may study them electrophoretically

Wright Thank you very much Dr Seegers That is very interesting

Dr Best you have some comments?

Best I think it would be very good strategy to ask someone who knows as little about this subject as I do to make some comments at this time

I think if we look over the record we might be accused of saying yesterday a lot of the same things we were saying last year sometimes almost word for word Certainly this cannot be said about what has been presented this morning and I think these presentations mark really a very definite step forward I am hopeful that perhaps Dr Baer s work in my department might be of interest to this group in the not too distant future He has synthesized hydrolecithin If he can substitute an unsaturated fatty acid for the saturated ones that he now uses he will have the lecithin which we all know This might help us to unravel some of the complexities of these problems we have discussed

Flynn We will certainly try it

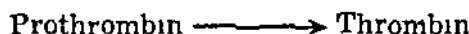
Since we have been able to activate purified prothrombin artificially it seemed advisable to determine whether or not we could not block the activation with simple organic compounds We discovered that 3, 4, 4 triaminodiphenyl sulfone, will block the activation of prothrombin by 30 per cent sodium citrate

Edsall At what concentration?

Seegers The compound 3 4, 4 triaminodiphenyl sulfone is very sparingly soluble, hence we use a saturated solution which involves only a very small amount of material What we are now doing is trying to vary the substituents in the molecule to see which compounds will act as blocking agents and which will not act as blocking agents, and we already know one of them which will do it very well—2 hydroxy-4, 4 diaminodiphenyl sulfone This compound also is sparingly soluble and we use a saturated solution

I wanted to make some comments as to the probable meaning of these experiments One point which I believe we have now made (at least I am convinced) is that prothrombin contains all of the necessary amino acid building stones required to obtain the thrombin molecule You do not have to add thromboplastin You do not have to add Ac globulin I doubt that you need to add calcium or anything that one might wish to suggest as an activator of prothrombin

As Dr Flynn said, these substances can be classified as accessory substances and I believe it quite definitely shows that to talk about calcium reacting stoichiometrically with prothrombin is out of the question I believe a proper way to write the equation for activation of prothrombin is as follows



Then we may add to that as accessory factors calcium Ac globulin, thromboplastin and, to satisfy anyone, X

Quick You probably need that X

Seegers I think when we add another X we ought to prove it though It seems to me that you could write in place of X now most anything including sodium citrate I would also like to make a point of the fact that I believe this helps very considerably in orienting our thinking particularly at a time when we are closely in danger of being very much confused not only by switches in nomenclature but also by *ambiguous nomenclature* I want particularly to stress that I do not believe there is any point in

in human plasma with the one stage assay merely means that the body is well adjusted with regard to these variables—obviously the inactivation of thrombin is more or less the same from one patient to the other the rate of formation is more or less the same the total amount of prothrombin present in the blood is more or less the same. Constancy can be obtained with the one-stage test but mere constancy does not give knowledge regarding any particular variable When conditions are radically changed as they are in disease or when they are changed by shifting from one species to another the whole problem breaks wide open Dr Flynn s method dissects the problem analytically separating the variables as the two-stage technique does routinely

In a busy clinic one might want to adopt a more practical measure for most cases Yet a fine analysis of specified groups cannot be done by studying gross mixtures which vary in an un known manner

Dr Seegers has presented interesting work I like to think of the problem of accessory factors and inhibitors as being wide open As Dr Best has said I have become more and more certain the field is an expanding field In fact we are just beginning to scratch the surface and dogmatism is particularly out of place

Wright Thank you Dr Smith Are there others who would like to comment on Figure 21 while it is up here? Dr Quick do you have any comments?

Quick No later I shall present certain things on the board

Edsall I should like to ask further about the question which I think Dr Flynn raised regarding the possible influence of anti thrombin in causing an error in the two-stage assay of prothrom bin The effect of antithrombin is generally minimized by greatly diluting the plasma before the conversion process is started Of course even after dilution the ratio of prothrombin to antithrom bin is still the same as it was in more concentrated solution but at the higher dilution the antithrombin is probably much slower in exerting its action so that the error involved is less This re-quires that there should be plenty of the factors needed for rapid conversion so that the rate of formation of thrombin is maintained at a high level while the neutralization of antithrombin is dimin ished by the dilution However while this greatly diminishes the error it is still hard to get away from it altogether I should like to ask Dr Flynn how great an error they think this factor does

Best In partial preparation for this meeting, I reread your review Dr Smith in 'The Annual Reviews of Physiology and I take it that even after going over all the literature you were still a little bit uncertain about the interplay of these various accessory factors I suppose it is obvious to suggest that, after isolating the factors you can go on studying them in what is not really a physiological system but an artificial one or that you can study them physiologically without being able to identify them The final answer will only come when all the factors have been isolated and recombined to form a physiological system

Smith I think Dr Flynn has done some interesting work in trying first of all, to go back and repeat the work of other laboratories We all understand that factor V or accelerator globulin, is a convertibility factor yet it has been known through the work of Owren Seegers and others that the amount of thrombin formed is also a variable Dr Flynn seems to have concluded that the ultimate levels of conversion rather than the mere speed of conversion is more important for the purposes of assay After all if the reaction is going to end at different levels, it will probably have different speeds in getting there This perhaps, is an important corollary

Also I think his efforts in bringing the two stage to bear upon analysis of the one-stage technique is a valuable contribution I wish the chart showing the two-stage analysis of the Quick test could have been before us yesterday when we were discussing the question of a standardized thromboplastin and whether the one-stage technique gives a reliable measure of the amount of prothrombin

Wright Would you like to have the chart on the screen again?

Smith In Fig 21 * we see that during the 28 second period the prothrombin falls off rapidly, at the same time the thrombin rises The lower curve is the rise of thrombin the upper curve is the fall of prothrombin To say that the fifteen second clotting time measures merely the 300 units of prothrombin without taking into account the way in which prothrombin is converted or thrombin is inactivated is to overlook the essential nature of the problem We are dealing with nicely balanced variables of enormous magnitude We have known for some years that something like this occurred We had verbal expressions of it, but never before have I seen such a complete analysis of one-stage assay The mere fact that one gets 12 or 15 seconds regularly

* Page 155

Seegers As I understood the question was whether or not lower forms might have quite different systems Dr Glavin of Copenhagen Denmark has done extensive studies with lobster blood which contains fibrinogen but the fibrinogen is entirely refractive to the action of mammalian thrombin An entirely different mechanism operates for jelling the fibrinogen No one has followed through on your question whether they do have accessory factors of an entirely different kind I presume that to answer your problem one would have to set up a group of workers of the caliber that we have here and put them to work for as long as they have been on the mammalian problem

Smith Dr Warner and I did some work years ago to show that the prothrombin level in the plasma undergoes an evolutionary increase from the dog fish on up to the mammals Fibrinogen is present in roughly undiminished amounts as one goes down the scale Concerning thromboplastin, I know nothing about it quantitatively in these lower forms As far as I know no extensive studies have been made of lower forms in regard to accessory factors like accelerator globulin

It appears Dr Fremont Smith that as one goes down the evolutionary scale the thing to disappear first is prothrombin

Seegers We published an article recently on Ac globulin in various species The number of molecules of Ac globulin per molecule of prothrombin is the lowest in the human The human contains less Ac globulin in proportion to his quantity of prothrombin than any other species

Smith Dr Flynn did some work on mammals and Dr Quick published work showing high values of the labile factor in the dog

Flynn Our results were very comparable to Dr Quick's and Dr Seegers work We have not published them

Ferguson I do think that species specificity in blood coagulation becomes highly significant in lowly forms like the invertebrates Indeed there is considerable evidence in the literature to suggest that some of these clotting mechanisms must be investigated primarily as an independent enquiry without *a priori* ideas that these are similar to the mechanisms in vertebrate bloods Dr Seegers referred to J Glavind of Copenhagen who has just published an excellent monograph on Studies on the coagulation of crustacean blood (1948) with a review of the literature which

introduce into an assay and whether more systematic methods for correcting it can be developed Perhaps the error is small enough so that *it can be safely disregarded*

Flynn I think Dr Smith is better qualified to answer the anti thrombin problem of the two stage We don't claim that the two-stage test is a perfect test Probably it can be improved upon in many respects Dr Smith did considerable work to show that anti thrombin plays a minimum role in the two-stage method

I think Dr Seegers points are well taken We are not opinion *ated as to the indispensibility* of the accessory substances for the test tube conversion of the prothrombin to thrombin They may very well all be catalysts It seems to me that the stage of mixing bloods is about over What needs more attention is the type of experiment that Dr Seegers has reported in which use is made of simplified systems purified reagents and parameterized variables

Would you comment on the antithrombin Dr Smith?

Smith I believe that the point Dr Edsall made namely that the titration is carried out in high dilution is the essential point When the thrombin titer rises to about the one-unit level, as it does after two or three minutes it tends to remain at that level for three or four minutes I would suspect that if anti thrombin were very important in the first minute or two it would continue to be somewhat important for the next two or three minutes That is not the case However I think there is no deny ing a little loss of thrombin in the two-stage assay by virtue of the action of antithrombin I have seen a slight falling off of the *curve and I have suspected that perhaps there was a 10* per cent or 15 per cent error I doubt if it is more than that There is a paper suggesting that alcohol incorporated into certain of the two-stage reagents was of value in eliminating the effects of anti thrombin

Ferguson The paper you made reference to is [L A Sternberger *British J Exp Path* 28 *168 (1947)*]

Fremont Smith May I ask whether there is a lower form of life containing a thrombin fibrinogen system but with fewer acces sory factors? Is it possible that in the evolutionary process the accessory factors have come in later? We might gain something from the simplicity of working with such a system provided of course it exists

our own to see if we get the same kind of results We ourselves have a few points of difference in our experimental work, which I should like to discuss

Thrombin Yields and Thrombin Stability

The data concern thrombin formation from a number of Dr Seegers purified prothrombin preparations [Blood the J Hematol 3 *1130 (1948)*] Our prothrombin solutions are made up in a simple borate buffer pH = 7 7 and specific resistance 170 ohms (21 C) The mild bacteriostatic powers of the borate are valuable in some tests extending over several weeks at room temperature The buffer is used as diluent to constant volumes throughout and as solvent for all organic reagents In a series of tests a constant amount of prothrombin solution is treated with the various activa tors (and inhibitors) we wish to study Measured samples of the thrombic mixture are removed after various periods of incubation and the clotting times noted on adding them to a standard test fibrinogen The clotting times afford a relative measure of the thrombin yield and may with some reservations be converted into percentages (of optimum) by reference to a dilution series pre pared from the fully activated mixture All possible controls are run especially of the second phase to detect any inhibitory or clot aiding (fibrinoplastic) efforts This technique differs from the Iowa two-stage method in (1) avoidance of dilution variables (2) non use of acacia (3) minor differences of buffer and reagents and (4) desire to steer clear of any claim as to absolute *units* since we are still inclined to distrust the empiricisms on which these are based

A significant experimental divergence from the Iowa method is the *incubation* period required for reaching optimal thrombic potency Drs Smith Brinkhous Seegers and Flynn always get an end point of maximal thrombin formation in about 3 5 minutes under optimal activation conditions Even with prothrombins strong enough to give an optimal clotting time of 4 5 seconds we seldom need less than 15 min and often longer if the prothrombins are weaker etc A second point is that in the Iowa technique there seems always to be a lengthening of the clotting times after a few minutes of incubation whereas we have many experiments (*ref* cited) to show an amazing stability of our thrombin optimum usually extending over 3-4 weeks at room temperature The third

is very valuable His experiments were performed chiefly on lobster blood, in which, as in several other crustaceans " the same coagulation enzyme was found which had nothing in common with any of the enzymes acting in the coagulation process of vertebrates ' the designation *crustacea coagulin* (*cf* L Loeb 1903) is proposed

Last summer I did a few preliminary experiments with the clotting of crab blood and came to the same conclusion Nothing in crab blood would clot human or bovine fibrinogen and the crustacean clotting was not affected by mammalian thrombin or thromboplastin

Fremont Smith The question might be raised whether the time and effort concerned in working out some of these different and more primitive systems might not throw light on the system that we are concerned with more rapidly than a continued preoccupation with the single system I think there is a scientific historical reason to believe that that kind of an approach is often enlightening particularly as some of the elements in the several systems are identical

Wright Are any of them identical so far as is known?

Fremont-Smith Fibrinogen is present as far down as the crab

Wright Is it identical?

Fremont Smith Identical' is perhaps wrong, closely analogous I stand corrected

Edsall We had some discussion last year about the horse shoe crab and there the clotting material does seem to be very different from the mammalian fibrinogen

Ferguson The major investigation I shared with Dr W C George last summer was on the blood of the large conchs (Busycon spp) This, unlike crab blood had no clotting property and did not even gel' like Limulus blood The only phenomenon was an agglutination of the amoebocytes without significant disintegration Observed *in vivo* this seemed to have no hemostatic function since it failed to adhere to or plug up the puncture wound in the blood vessel Protected by the armor of its shell this mollusk would appear to have little if any need for a hemostatic mechanism

Ferguson I would like to record how impressed I am by the very interesting presentations this morning I do believe we shall all consider them very carefully and conduct similar experiments of

to explain the divergence The only significant instability we have found in antithrombin free and protease-free mixtures occurs at high thrombin dilutions The minor differences in the last two columns (Table 22) could be experimental depending upon the time elapsing between making the dilution and testing [*ref* Blood 3 *1180 (1948)*] We have a few recent data suggesting that this deterioration of very weak thrombins is slower in silicone tubes but we want to do some more work on this I do want to raise the question whether by use of high dilutions and the introduction of acacia Dr Seegers (et al) are not introducing some insufficiently explored variables having independent effects on thrombin yield and thrombin stability I would like to ask Dr Seegers perhaps to answer later in connection with what he was telling us this morning about the effects of incubation with citrate whether he feels that a purified prothrombin solution may *always* undergo spontaneous activation even though it may take days or weeks rather than hours to estimate the extent of this From the variability of activation times spontaneously or on addition of calcium alone etc we prefer to think that even the best of Seegers prothrombins still contain minute but significant amounts of activator impurities (Blood op cit)

Going back to Dr Smith s comment that in plasma prothrombin assay the thrombin optimum must be taken as the lowest point of a curve in which activation is succeeded by inactivation I wonder whether the Iowa method is not to be criticized for failing to secure a thrombin which holds its optimal potency over quite a long period How otherwise can we know how much thrombin loss occurs even in 3 5 minutes *during* the activation period? Further may not such an optimum represent not complete prothrombin conversion but an equilibrium point where the two simultaneous processes of activation and inactivation are proceeding at a balanced rate? It is considerations such as these that lead us to question the thrombin unit

What activators you must add in a prothrombin assay is a very important question Even a mere accelerator must be significant in the balanced activation inactivation system just discussed Dr Seegers and his colleagues are pretty certain that Ac G is merely an accelerator and Owren s data were similar Dr Lewis and I have recently [*J Clini Invest 27 778 (1948)*] made a very painstaking effort to get rid of the last possible traces of Ac G by vigorous heating of prothrombin and thromboplastin The loss

point is that under conditions of poor activation sometimes "spontaneously" and frequently with the addition of calcium alone, the ultimate thrombic potency even if it takes several weeks for completion, is identical with that obtained in a matter of minutes (see above) under optimal activation conditions Drs Smith and Seegers have been somewhat critical of this in suggesting that our clotting times were too short to permit the detection of perhaps considerable thrombin differences This doesn't seem a valid criticism since we have no difficulty in increasing the clotting times in the (final) thrombin dilution series In the following experiment, (Table 22) I believe we have a convincing refutation

TABLE 22

Dilution series Clotting times (for fibrinogen) in seconds at room temperature for the *relative* thrombin concentrations ("percentages) noted

Mixture	100%	50%	25%	10%	5%	1%
I	4	8	11"	22	40"	84
II	4	8	11"	22"	42	102"

Mixture I was completely activated in about ½ hr with added calcium and brain thromboplastin The dilution tests were made on the third day

Mixture II was the same prothrombin solution which had activated spontaneously on keeping in the icebox over a period of nine months During this time the refrigerator was defrosted several times, so I m sure the conditions were not ideal for storage Before making up the dilutions, enough buffer solution was added to equal the volume of original prothrombin plus activators in mixture I

The similarity of the two series of tests is remarkable and must be taken to mean very close equivalence in terms of thrombic potency

We feel strongly that highly purified prothrombin and thrombin solutions have very great stability In any system which indicates otherwise we should look for an additional factor or factors

up in our notes but I don t recall at the moment I am inclined to think it did not work

You wanted to know whether prothrombin that had been set aside for a period of about ten months would become active. I do not have any idea since we have not tried it I do think it might be well to put some in various solutions and sterilize them and set them aside and see what they look like at the end of a year

Overman I would like to ask Dr Seegers if he has tried sodium citrate at various concentrations on whole or diluted plasma?

Seegers We have not done any experiments whatsoever on whole plasma

Overman I am interested in the fact that Dr Seegers has been able to activate a purified system of prothrombin since this has been one of our problems with plasma We have been able to activate prothrombin in diluted blood and diluted oxalated plasma but not in whole blood or plasma This might be explained by the fact that the antithrombins are more active in whole plasma than in diluted plasma as Dr Flynn showed on his graph Dr Flynn have you carried out this same experiment using the diluted plasma technique? I think it would be interesting to find out the effect that these other factors have on the diluted plasma method

Flynn No we have not analyzed the diluted one-stage technique but it is a good suggestion and we could try it without too much difficulty

Overman In a matter of minutes we have been able to clot diluted blood and diluted oxalated plasma with the things that I have mentioned In our work on the purification of the inhibitor from soybean phosphatide we have isolated a fraction which will also clot diluted oxalated plasma without the addition of calcium and thromboplastin So I think it is very important to consider all of these possibilities when we are dealing with the activation of prothrombin

Seegers I hope that these experiments we are now doing will serve in a way of orientation by showing if you are satisfied that they do that prothrombin can be activated without accessory factors To reiterate there are in the prothrombin molecules all the necessary ingredients for the thrombin molecule The accessory factors need contribute nothing to the thrombin molecule in the way of material substances They apparently furnish an environment as catalysts for the rapid activation of prothrombin

of considerable prothrombin complicates the data somewhat by reducing the best thrombin yield to 22 per cent of the original, but the control lacking the Ac G was completely negative I stress this point because the data are not as convincingly stated in the tables and figures of the cited paper as they might have been owing to the fact that we were working with a fibrinogen containing a trace of prothrombin In Table VI of the cited publication we could reasonably have ignored this and put zero" instead of 0 5 of a "unit" in the data of the last line

Among the possibilities of interfering agents in plasma and other materials in which we want to measure thrombin I should like just to mention the plasma protease system(s) Activated plasma protease readily attacks fibrinogen and fibrin and we are convinced that it can destroy thrombin, also, if the enzyme is strong enough, stable enough and acts for a sufficiently long period [Blood 3 *1130 (1948)*] At the time we thought we were confirming Dr Seegers finding that the protease easily attacks prothrombin, there was no thought about the possibility of what might be happening to the 'accelerator" globulin We have recently studied this and found Ac G to be probably the most susceptible of all the clotting proteins to destruction by natural plasma protease (plasmin or fibrinolysin) In some of these new experiments with added protease, the prothrombin was rendered inactive (to optimal calcium and thromboplastin) but recovered the ability to give the original thrombin yield when Ac G was subsequently added Simple incubation of the accelerator globulin with enzyme readily destroyed the Ac G It is suggested therefore that it is the Ac G rather than the prothrombin which is attacked by the protease We are at present learning the tricks of the Iowa prothrombin assay and hope shortly to have experimental data which will include some observations on whether or not the natural plasma protease may be a factor to be considered in running plasma prothrombin tests

The antithrombin and heparin inhibitory systems should also be mentioned I would like to ask Dr Seegers if he used heparin at all to try and inhibit the activation of prothrombin by citrate?

Wright I think we had better have these questions answered now as much as we can Dr Seegers I think one or two of them were directed at you

Seegers I don t recall Dr Ferguson whether we have tried heparin or not We tried very, many compounds I could look it

Fremont-Smith For communicative purposes

Wright I believe that this morning we have had several examples of the need of more specific use of words Francis Bacon was apparently very self-critical in this regard and to whom should we look for a better example?

Flynn There is one supplement I would like to add One time Dr Smith and I were discussing problems of communication He made a point that was a good one I think there would be considerably less lost in communication if everyone took pains to repeat another man s experiments *I believe that particularly* applies in blood clotting

Seegers I would like to make a comment which I believe is necessary to supplement the remark I made previously The following facts were given one factor V is absolutely necessary for the activation of prothrombin under physiological conditions Two the activation of prothrombin does not require Ac globulin because it has been demonstrated that prothrombin contains all of the amino acid building stones to give the thrombin molecule I then made the statement, therefore we can say that these accessory factors do not become a part of the thrombin molecule That has to be modified a little further One should say provided the activation under physiological circumstances yields the same thrombin molecule obtained under my conditions

Wright The words have now been made completely subservient to the subject

Ansely I would like to throw a few rocks here sooner or later if I might The meeting is very amicable at the moment Hence no one can claim I am throwing rocks specifically at him You had a poem at your last year s meeting Just for a start here is one for this year s

There was a man in our town
And he was wondrous wise
He could unscramble scrambled eggs
And uncuss custard pies

And there is another verse which says

There was a man in our town
And he was full of custard pies
When they unscrambled him my dear
The parts they found looked very wise

(No bitterness at all in that)

I had some very interesting and pleasant visits with Dr Owren last summer Throughout his monograph he says that factor V is absolutely necessary for the activation of prothrombin, and perhaps there are a few other statements in there in which he says that something is absolutely necessary for the activation of prothrombin That is in conflict with our work showing they are not necessary In the literature one finds many such antagonisms Now I don't like the trite phrase that they are both right because I think in the end there is only one right view on anything but there is a twist to this one Owren did demonstrate that factor V is absolutely necessary for the activation of prothrombin *physiologically* because if it is not there bleeding results Just because one can activate prothrombin in a purified system with no accessories present, does not preclude the fact that these activators are necessary for physiological hemostasis

Fremont Smith There is a tendency in practically all disciplines to fail to specify the conditions under which the words *necessary absolute* or *always* are used Considerable controversy could be avoided if we used some such phrase as with respect to" whenever broad statements or generalities are made I believe the remarks you just made, Dr Seegers pointed this out very nicely

Wright To emphasize that the problem of communication was recognized by Sir Francis Bacon I might mention that this very morning Dr Flynn and I were reading from the first edition of Sir Francis Bacon which contains the ' Essay on Life and Death (1659) —which I have the good fortune to possess In the paragraph in the first section dealing with Bacon s life we encountered, nearly 300 years later William Ranley s statement regarding Sir Francis as follows *In the composing of his Books he did rather drive at a masculine and clear expression than at any fineness or affectation of phrases and would often ask if the meaning were expressed plainly enough as being one that accounted words to be but subservient or ministerial to matter and not the principal And if his stile were polite it was because he could do no otherwise Neither was he given to any light conceits or descanting upon words But did he ever purposely and industrially avoid them For he held such things to be but digressions or diversions from the scope intended And to derogate from the weight and dignity of the stile'* These words might well be taken as a guide to Josiah Macy, Jr Foundation Conferences—and to all scientific meetings

Kniseley At least three sets of definitions of blood clot are possible today a morphological descriptive definition an operational definition and a specific chemical definition

Our ideas on the structure of matter are organized on several levels of complexity One can think about electrons or other structural constituents of atoms or about whole atoms or about molecules or complex groups of molecules clear up through various stages of polymerizations of groups to aggregates the size of complex protein molecules Or we can think in terms of blood platelets or of cells and of groups of cells making up a whole tissue etc

Most morphological descriptions of clots are based upon observational study of the completed clot Clots have been studied at the low magnifications of the microscope and at higher magnifications up through the oil immersion Such morphological descriptions of clots may provide descriptive definitions and include such terms as platelets cells and fibers Such descriptions and definitions can be extended in detail by studying the clot with dark field phase microscopy and electron microscopy and perhaps some of this has been done But all such studies will probably yield only more detailed morphologic descriptions of clots They can of course identify the cellular elements within the clot but they almost certainly cannot analyze the chemistry of the substances which went to make up the clot

The operational definition of clot which has been most in evidence thus far at this meeting consists in stating that the experimenter had a tube containing one or more substances and that when the tube was held upside down the contents of the tube did not run out and was therefore clotted

Note that this method of identifying clot will not necessarily select for us the same set of clots as we might select by applying the aforementioned morphologic descriptive definitions Note that this operational definition merely states that something did not run out of the tube And thus many *different* complex mixtures may be present in a series of these experimental test tube clots

The specific chemical identification of the substances which go into the clot forming reactions are of course one set of objectives of the studies of blood clotting going on during this generation of men From the studies and discussions presented at this meeting it is most obvious that this set of chemical definitions is far from complete

When you wrote and asked me to come here, I was quite sur prised for I don't know anything specific about blood clotting

The first student paper which I tried to write in elementary physiology was on blood clotting I found that there was a terrific terminology and that it was necessary to acquire and learn the terminology (But when that was done I wound up with very few real ideas) So I would almost beg the conference to consider making a definite glossary of the current blood clotting terminology

You people all know blood clotting very well But, a new man coming into the field finds that when someone uses a certain set of words the specific terminology often partly overlaps the generic terminology Some of the specific terminology here relates to specific chemical substances One such substance has been chemi cally defined and you even know the length of its molecules Other words are here being used as a radio announcer might use them as though those words referred to specific substances when actually, as far as I can tell the only identification of the material referred to has been by way of a laboratory operation Having gone through a manipulative operation we come out with a material If enough investigators go through somewhat similar but not necessarily *identical operations, then this "word and whatever it refers to* come to have the *value* in subsequent thinking of a single chemical substance There ought to be a double terminology schedule, one part operational, the other referable to specifically identified chemical substances

Professor Best ticked me off pretty hard on his last year's first question 'Why does blood not clot in vivo? Professor Anton *Carlson also always used to start off his presentation and discus*sions on blood clotting with that question All my assumptions started that way too until I began watching some living traumatized tissues under the microscope When living tissues are crushed blood flowing through vessels in or near the crushed area changes from its normally fluid state to a series of semi solid red cell con taining, gelatinous masses (Cf Knisely Bloch and Eliot 1945) That is, under some circumstances not yet completely defined blood does perhaps 'clot in vivo

As a somewhat different point of attack let us take the word 'clot" What do we really mean by the word 'clot'?

Wright I was going to ask you for a definition

a needle over it A much larger needle is used than the ones which Professors Chambers and Zweifach have used as micro-needles The needle we use is a zoologist s insect pin It may be drawn over the tissue so that it passes across the blood vessel or much better so that it is drawn beside the blood vessel but does not touch it If the needle is drawn *over* the blood vessel the blood in the vessel may thrombose the vessel instantly The red cells in the vessel are trapped and held in a rapidly forming jelly which instantly plugs the whole vessel If one crushes the tissue to one side of the vessel the response is somewhat more complicated Fluid blood containing unagglutinated red cells comes in on one side of the crushed territory and some one or more gelatinous substances form around groups of these red cells as they pass through the vessel near the crushed tissue These gelatinous substances cement groups of red cells into semi rigid masses which pass off in a row down stream into the general circulation This reaction is now known to occur in mice rats cats guinea pigs dogs and Rhesus monkeys And we are pretty certain that it happens in human beings because we have seen gelatinous semi rigid masses show up in the small vessels in the side of the white of the human eye following severe contusions of the leg Hence we believe that the reaction is a somewhat general one This kind of reaction occurs in tissues of frogs also but the sludge formed in the frog is somewhat different from that formed in mammals because the masses are softer and seem more flexible

If these masses are clots and no sophistry is intended here —if in terms of chemistry physics and histology these masses are clots—then blood can be made to clot in vivo by this kind of crushing injury to tissues near small blood vessels

One might wonder why this reaction has not been discovered and thoroughly studied before this The failure to find the reaction is probably because of the methods for studying tissues which have commonly been used Most of them probably do not find the agglutination or perhaps we might even say clotting of the blood flowing through vessels near crushed tissues For example if the reaction is initiated in mesenteric veins the small masses formed *in the region of the crush are carried to the liver and under* some circumstances at least the sticky materials containing red blood cells are phagocytized and that very rapidly If one cuts histologic sections of liver one probably would not find much of the material or red cells in hepatic phagocytes because such phagocytes

So here we are attempting to study the process of clotting' while partly using three different definitions of a "clot' no one of which shows much sign of being complete or satisfactory

One can only hope that by noting the different ways in which we are using our words it may become possible to clarify each type of definition and thus make it more useful for comprehensive understanding

May I turn the discussion at this point to the subject of thrombosis? During this meeting there seems to be an underlying assumption to the effect that somehow the clotting mechanisms under discussion are pretty tightly related to thrombosis This assumption partly contraverts one of my favorite prejudices One cannot guess physiology from anatomy And one cannot guess pathologic physiology from normal physiology One can make hypotheses and test them but one cannot extrapolate without test from one field of biologic knowledge to the next Hence I fear that if we build up backgrounds of knowledge of normal physiology and then without conscious awareness, look at our patients and get samples from our patients and extrapolate our patterns of thought about normal physiology into crystallized patterns of thought about pathology we are going to miss an awful lot of pathologic physiology Pathologic physiology may and almost certainly does contain a great many factors of anatomy, physiology and chemistry which do not show up in normal anatomy physiology and biochemistry

If I may steal a few minutes I would like to show you quickly on the blackboard two or three kinds of phenomena which we have been seeing They have had meaning to us and may have some new and different kind of useful meaning to you We desperately need help toward explaining the observations and understanding them

On the question of blood clotting in vivo, may I discuss what we have seen and later argue whether or not they are clots (a large Y' shaped vessel was drawn on the blackboard) Suppose we take a little vein like this 'Y shaped one in mammalian mesentery with the blood flowing through the vein in such a direction that the two streams in the branches come together forming one stream and then enter and pass downward through the stem vessel We can use the small vein on the left side for a control and do experiments on the right side The tissue on the right side next to the small branch vessel can be crushed by rubbing

If a bit of tissue is crushed at a distance say of 100 micra from a small vein the reaction begins within the vessel almost right away If one takes a motion picture through the microscope of this reaction to crush beginning right away counts the frames — —

Barker What do you mean by right away?

Knisely A quarter of a second which is a long time When you take a motion picture at 24 frames a second each frame will be exposed approximately 1/50 of a second and 1/50 of a second elapses between the frames Motion pictures have been taken at these rates which record both the crushing of the tissue and the beginning of the formation of semi solid masses within the vessels

The first visible response which one catches in a motion picture is about ¼ of a second after the crushing This may mean merely that if we had a very high speed motion picture camera we might catch the beginning of the reaction earlier The reaction may begin at least as soon as ¼ of a second after the tissue is crushed

Blood may change from a completely fluid state with un agglutinated cells to a thick sludge in from ⅛ of a second to a second The rates of the reaction have been measured by counting the motion picture frames during which individual red cells move from a given position in the tissue to new given positions in the tissue during which time and transit they are incorporated in precipitates which form around them (cf Knisely Bloch and Eliot 1945)

Now for the duration of the reaction Crushing injury to the tissue can initiate the previously described process which may then go on continuously for as long as from 4 to 8 hours

During all the above descriptions the blood in the vein on the left side of the small 'Y shaped drawing on the board remains entirely unagglutinated This is a control which indicates that precipitation of the blood passing through the right side is due to the crushing injury

There are precipitates between and around the red cells which hold masses of red cells into cohering solids One can see the cohering solids turn and tumble over and over as they are carried along down into larger and larger venules

To find out if chemical substances might be coming from the crushed tissues diffusing in through the vessel walls and initiating the formation of precipitates in the passing blood the following

digest red cells very rapidly [Knisely, *Anatomical Record* 65 *131 (1936)*]

If one were to draw a sample of blood from the general circulation one might easily not find the small masses particularly if the volume of crushed tissue in the subject was rather small The reaction occurs rapidly in the crushed tissue, and the masses might either be phagocytized and thereby very rapidly removed from the blood in spleen, liver and bone marrow or, if but few masses were formed they could be diluted or scattered into large volumes of blood rather rapidly

To return to the original theme, if these masses were clots then blood can under some circumstances at least, clot" in vivo One of the amazing things is the speed of this whole set of reactions

Best There are no platelets in that?

Knisely I do not know all that is in it, but certainly each mass contains no more platelets per unit volume than there are in circulating blood The speed of the reaction precludes the possibility that platelets are carried in and localized within the injured vessels before the fluid contents of the vessel solidify

Now, I would like to talk a little more about the speed of these reactions The astonishing features are the speed with which the reaction is initiated the short length of time it takes to form individual masses and the duration of the periods while these reactions continue Certainly for experimental purposes, it is perfectly legitimate to put things together in such concentrations that the durations of the reactions are long enough for the experimenter to study each part of the process However, it is necessary when studying pathologic physiology to begin to learn the actual rates at which chemical reactions occur in the living body

Tocantins How large are the small vessels in which these reactions occur?

Knisely Anywhere from 20 to 200 micra or somewhat more in diameter

Tocantins Work on blood clotting is usually done in tubes about 10 mm in diameter so the times cannot be compared with the rates of change in those living vessels

Knisely I am not being critical of the chemical studies No sophistry is here intended

capable of (1) diffusing for short distances in short periods of time (2) diffusing in through vessel walls and (3) initiating the formation of sludge in the blood passing through the undamaged vessels

At present we do not know how long such substances persist in such a pulp but this can soon be found out, and probably will have many answers depending upon the species of the animal the tissue used, etc For myself, I am deeply curious to find out if such substances can come from some of the more common types of pathological lesions which are present and occurring within living human beings (For example do the lesions of pulmonary tuberculosis continually release substances capable of agglutinating the blood passing near them? Agglutinated circulating blood has now been seen in more than 50 human tuberculosis patients in the Chicago Municipal Tuberculosis Hospital (cf Knisely Bloch Eliot & Warner 1947) and the high sedimentation rate of blood from tuberculosis patients is well known)

To return to the experiments when bits of crushed tissue from the above organs are placed in the surfaces of mesenteries which are very thin the reaction begins in the visible vessels at the edge of the pulp in approximately ¼ of a second to a second

The impressive feature of this experiment to me is that there may be a great many substances in crushed tissue and there is no reason at present to assume that but one or two of them are capable of initiating reactions in the passing blood Nothing as yet has been isolated from these tissues which is alone capable of initiating the reaction—probably not because of difficulty but only because we have not yet been at it The feature which worries me is that there may be many more things present in crushed and otherwise injured tissue than the list of known chemical entities which have been discussed at this meeting but this may well be the nature of some kinds of pathologic physiology When my boy falls off his bicycle and bumps his knee the reactions in his blood may be much more complicated than the chemistry of isolated clotting

A couple of other observations may interest you An experiment which failed taught us a lot In early phases of the work when small bits of crushed tissues of brain (each about ¼ the diameter of the head of a common pin) were placed over mesentery we found that sometimes the blood flowing underneath did not form the solid precipitates Later we found that this occurred where the blood was flowing most rapidly When the area under

experiments have been done Two animals are used for each experiment One is anaesthetized and his mesentery prepared for microscopic observation as above The operation is done without losing any blood and by techniques which minimize or prevent the formation of sludge (cf Knisely, Bloch and Eliot, 1945) The other animal of the same species is killed, either by a blow or by cutting off its head Individual tissues or organs are taken from this animal such as bits of liver, spleen, striated muscle smooth muscle, brain testis etc Each kind of tissue is placed by itself between two glass slides and crushed A bit of this tissue pulp from a single kind of organ is then placed on or near small vessels in the mesentery of the previously prepared mouse at a point where blood can flow within the mesenteric vessels very close to the surface of the mesentery The bit of pulp is about ¼ the diameter of the head of a common pin With such a preparation the blood flowing through the small vessels can be observed before, during and for long periods while various substances (and their inhibitors) are placed in close proximity to the vessel walls The reactions of the blood can be studied without complications which might be introduced by withdrawing it from the body

When tissue pulps from any one of the above organs are placed beside small vessels the blood flowing through the immediately adjacent vessels begins to go through the previously described series of reactions — —

Quick You get a reaction or not?

Knisely You do Precipitates form in the flowing blood, changing into a sludge The experiment has to be done with a few precautions One has to be careful to put the tissue pulp very near to the vessel For example if one puts the tissue pulp on the outside of the small intestine it is necessary to put it directly over a vessel which is immediately beneath the outer surface of the small intestine so that the substances from the pulped tissue need not diffuse for a very great distance

This type of experiment can fail for several known technical reasons one being that the pulp is put too far from the vessel being observed Thus far the time between the killing of the animal which donates the pulp and the time the pulp is put on the tissue of the living animal has always been kept as short as possible usually within 5 or 10 minutes

From the above type of experiment one may conclude that one or more substances are released from crushed tissues which are

through mesenteric vessels slowly, but no visible precipitation appears in closely adjacent vessels of the same kind and diameter in which the blood is flowing rapidly

One other concept which interests me here is as follows The rate of rotation of the fibrinogen molecule is approximately 100 000 times a second And one thrombin molecule makes approximately 10 000 unions before becoming inactive The rates of these 100 000 a second and 10 000 unions a second can happen so rapidly that our method of making motion pictures to measure the rates of chemical reaction begins to look pretty foolish further a quarter of a second begins to look like a very long time

Best When the blood flow is speeded up is it your point that nothing happens or just that you don t see what happens

Knisely We just do not have any way at present for making this distinction We are faced with the limitations of the human eye as a tool for observing

Fremont Smith You mean they are washed away so fast you do not see them?

Knisely Yes that is possible Particles may be so small that they are not visible in flowing blood At some of the currently used magnifications it might be necessary to have masses 50 micra in diameter to be observed in flowing blood This statement fits the situation exactly Masses may be present but too small to be observed

Edsall If the blood is flowing fast and the material entering the blood from outside is flowing in at a constant rate the amount which enters per cc of blood flowing by is smaller than if the blood is flowing slowly Perhaps this has a good deal to do with the effects you observe

Knisely We are exactly at the point where this kind of observation gives only negative evidence Did we find that nothing happens or did we fail to find something which happens that is where this experiment breaks down

Tocantins A similar thing happens with injections of tissue extract intravenously If one has a blocked segment of a vein and injects the extract into the segment one gets clotting im mediately of the blood in the obstructed vein If you inject the same extract at a slow speed into the free circulation there is some deposition of fibrin but the circulation remains open

Knisely Yes

some tissue pulp contained vessels of similar sizes, some with fast flow and some with slow it was found that the reaction occurred in the vessels with the slow flow but not in the vessels with rapid flow This almost certainly was because solid masses were not formed in the rapidly flowing blood and hence there was nothing at which we could look to know that the reaction was occurring, rather than the alternate possibility that no substances were re leased or that no reactions were occurring (vita infra)

Chemistry, as ordinarily studied and taught today, ofttimes blindly assumes that most things within mixtures are free to diffuse or to be mixed in containers by stirring and swirling In most places the rates of bringing reacting ions, atoms or molecules together depends upon such mixings and diffusings I do not know any physical chemistry concerning the thermal rates of movement of molecules But one of the major functions of living anatomy is to determine the rates at which certain sets of mole cules are brought together and this is sometimes accomplished by forcibly limiting the rate at which atoms or molecules of a given kind are brought into the field where the specific reactions can occur

Supposing we have a chemical reaction which can occur within circulating blood and which takes place between two and only two kinds of reacting units Suppose one comes down through the vessel in circulating blood, and let us indicate this one by a series of individual x marks Each 'x indicates one ion of this kind Supposing the other substance can come only from the tissue, and let us indicate each ion of this substance by a small o' Sup pose further that x" cannot diffuse out through the vessel wall into the tissue but that o can diffuse in from the surrounding tissue through the vessel wall and pass into the flowing blood Suppose further that the reaction pattern of these two is stoichiometric and that when one "x and one o" come together a reaction takes place *If the rate of flow through the vessel is* rapid many 'x s can pass a certain point per unit of time If the rate of diffusion of 'o s in through the vessel wall is slow then one "o arrives in the vessel for but a small percentage of the number of 'x s' going by, and many 'x s pass by which do not receive the necessary o s' with which to unite

There is *some* experimental evidence which agrees with this concept As noted above sometimes a particle of crushed tissue placed on mesentery initiates visible precipitation in blood flowing

obvious that we know so little that one emotional reaction is to go to work for 10 years Another is to get the small amount we know published and let a lot of other people go to work at the same time

In the past almost all microscopic studies of circulating blood have been made with the microscope in the vertical position with the observer looking downward To see masses settling out of columns of blood to the lower sides of nearly horizontal vessels we have been turning the microscope into the horizontal position so that the observers are actually looking horizontally A thin membrane of tissue usually mesentery is mounted vertically Some of the vessels in the tissue run horizontally or nearly horizontally The observer looks into the sides of the vessels and can observe masses of agglutinated blood cells settling to the lower sides

Perhaps I should start a little ahead of these observations to indicate why they may be important In some 5 000 healthy vertebrates including human beings the blood cells have been found to be completely unagglutinated Per contra members of our laboratory have now seen agglutinated circulating blood in more than 1 000 human hospital patients diagnosed by practicing physicians as having something more than 50 different pathological conditions and diseases Many different kinds of masses have been seen large ones small ones some which are very rigid and some which are easily squeezed out into spindle shapes as they pass through the cone shaped terminal arterioles In some patients all the masses have been fairly large and fairly rigid a pretty serious business Some patients have large masses which are relatively soft some patients have small masses other patients have mixtures of sizes some of which being large and sufficiently rigid to plug terminal arterioles permanently At the moment the total amount of human knowledge concerning what materials might stick red cells together into large masses seems to be quite largely in the fields of blood clotting and of immuno-chemistry Stats D and Wasserman L R [*Medicine* 22 *363 (1943)*] have brought together and reviewed a large number of papers on substances capable of making red cells stick together which may very well be related to the chemistry of the sticky precipitates which cause agglutination of human patient s circulating blood

Of substances in blood capable of sticking red cells together it seems most easily imaginable to suspect blood proteins And probably, the sticky materials which hold red cells together will

Tocantins The animal does not die If you inject the extract rapidly you overwhelm the circulation There is massive intra vascular clotting and the animal dies within a minute or so

Knisely Our experiment *is in strict agreement*

Some of you may have been thinking about phagocytosis how fast could coated masses of blood cells be removed from the blood stream. Some of the current immunologist's concepts are to the effect that particles cannot be selectively phagocytozed from the blood stream very rapidly But this certainly is not true [cf Werigo *Ann d l Inst Pasteur* 6 *478 511 (1892)*] Further as a classroom experiment we have taken a white mouse and in jected into it 2 cc of Higgins India ink every bit of which will go into the phagocytes of the spleen liver and bone marrow if the ink is injected slowly and steadily into the mouse s jugular vein for a period of about ½ minute As noted, this has been done in a class All the blood which could be obtained rapidly was im mediately removed from the mouse and the blood was put on glass slides and all of *it examined by the students Each student is told* to look for ink in the blood on his slide Thus far, no student has found any ink Further if some of the fresh tissues are put on *slides and students look for ink in the tissues very little is usually* found But if the liver and spleen is cut out and quickly cut into thin pieces and put on slides or if histological sections are made of these organs large quantities of ink are found in liver and spleen and all of this is found in phagocytes The significant feature here is that large quantities of India ink can be selectively removed from the circulating blood by the phagocytes of spleen and liver in very short intervals of time For some detailed statements see Knisely Bloch and Warner 1948

The living machine runs at much higher rates than we have been thinking of (If one has a good sharp class and if there are any holes in the above classroom experiment members of the class will find them)

Best This is the new anatomy

Knisely This is *anatomy trying to become physiology*

May I try to say a few things related to the problem of throm bosis I am a little full of this subject right now because we are trying to write a paper which can only tell how little we know If one writes a paper for an editor he is supposed to say that he knows something and I think we do know a few things But it is

Miss Louise Warner of our laboratory has been making the studies of the settling of masses to the lower sides of vessels [cf Warner *Anat Rec* 100 *No 4 Amer Assoc Anat 784 (April 1948)*] It is an outline of some of her work which I shall present in the next few paragraphs A few key points are as follows sometimes masses settle to the lower sides of vessels and do not stick to each other and do not stick to the vessel wall Loose masses rest on the bottom of the vessel sometimes for long periods of time just as glass beads might rest in the bottom of a beaker of water and never stick to the bottom of the dish

Sometimes after the masses have been on the bottom of a vessel for a time they begin to stick to each other becoming a much larger mass a small thrombus if you wish Sometimes the small thrombus does not stick to the lower side of the vessel so that upon a slight movement of the animal the whole thrombus is easily carried away down stream and it plugs up the next progressively narrow vessel it enters

Sometimes the large thrombus cements very tightly to the lower side of the vessel and cannot be shaken loose even if the tissue is experimentally moved quite a bit

One set of chemical questions now is why do the masses sometimes stick together making a thrombus but not attached to the inside of the vessel and why do they sometimes cement tightly to the inside of the vessel? Are there changes in the chemical composition of the plasma of the circulating blood even after the agglutination of cells has occurred which can cause the masses to stick together? Is this the only source of such substances or can lesions (perhaps microscopically undetectable lesions chemical lesions if you wish) cause the initiation of substances from around the vessels where the settling has occurred? Obviously there are a number of such possibilities

When such masses break loose familiar clinical situations of course arise Pieces may plug coronary arteries or cerebral arteries or renal arteries Miss Warner has motion pictures showing kidney *glomeruli of amphiuma becoming plugged up with masses which* are either large pieces of sludge or fragments of preexisting small thrombi

Perhaps this is the time to summarize and then cease and desist To you who are biochemists it is my privilege to ask

have to be analyzed by at *least* two classes of scientists, (1) biochemists and (2) immuno chemists In our current state of ignorance it seems that these substances may have to be described by each of these *groups of scientists in order to characterize* the substances

Perhaps sludged blood is partially clotted blood Yesterday I asked Dr Ferry *if it were possible for fibrinogen to polymerize* up to a certain stage and then these long molecules be prevented from further polymerization so that one might have blood which was partly clotted but not completely clotted So, I would almost beg you now to begin thinking of the possibility of having partially clotted blood or to have the clotting process arrested at temporary equilibria at various stages along the line toward large solid clots

There have been some observations which make this hypothesis seem somewhat attractive Following mechanical trauma to a dog for instance the animal s whole circulating blood changes to a thick pasty sludge, the masses of which for a time plug almost no small blood vessels In some animals each mass passing through a terminal arteriole changes from approximately a sphere to a spindle as it is driven down the long, cone shaped arteriole In some animals all the masses which have previously come down through the terminal arterioles undergo a change in internal consistency, becoming much more rigid and hard They change from soft to rigid and forcibly bulge the terminal arterioles which greatly *increases the resistance they present to pass through terminal* arterioles This change in internal resistance looks (perhaps) a great deal like the clot retraction phenomenon This is of course only a guess but it strongly suggests that the blood clotting reactions might stop at intermediate levels below the formation of a final large grossly visible, rigid clot

To take up the problem of thrombosis again The horizontal microscope focussed into sides of horizontal vessels were used first of all to study the settling reactions in rather small vessels, those from 50 to 400 micra up to a millimeter in diameter, etc

After sludged blood is passing horizontally in vessels of this size, masses frequently stick together forming larger masses When the masses arrive at a certain size they begin to settle toward the lower sides of the vessels In general they follow what one would expect from Stoke s Law

to perfect the technique and know just what to expect in the controls

Knisely It will be so simple to see the masses form and see them settle that you can put such experiments in any class in histology and any class in pathology The process of settling and cementing together which has been behind closed doors now will be as public as looking into Park Avenue windows

Wright Wasn t that explored by Laufman Martin and Tantini of Northwestern and reported in a September issue of Science?

Knisely I believe that one article has been published in Science

Fremont Smith I think this illustrates the preoccupation of one investigator with his own technique There are very few experiments or studies made with the combined biochemical and physiological approach In the first place the number of investigators in this country who observe living capillaries are exceedingly few The ones who do it limit themselves largely to the study of the capillaries seldom making an attempt to bring in an ancillary methodology such as chemistry The converse is also true with the biochemists ignoring physiological methods Obviously what is needed is a laboratory in which living capillary studies are made with a group of biochemists in the same environment to observe the same things and then to cross fertilize the problem with biochemical experiments It is this joint simultaneous approach the multidiscipline approach that is so essential to progress

Perhaps the problem is to be approached by having the biochemist learn the physiological technique or perhaps it is necessary to set up teams I think it will work better if a combined team of physiologists and biochemists are deliberately brought together to explore common purposes

Knisely Our work has been very slow in developing Early in our investigation there began to be more observations than we could keep catalogued One could put light into living animals in many places and see many new things At that time we were thinking like histologists Having seen dead sections we were looking at living tissues and living histology in terms of dimensions and changes in dimension during different functions Parenthetically I will add that is so different from dead histology that ultimately much of the current dead histology will consist of

what kind of substances might increase or decrease in the circulating blood during and *following various kinds of diseases and perhaps* after surgery How might such substances perhaps increase the threshold concentrations of substances which might cause agglutinated blood cell masses to stick together further so that they begin to build up into thrombi? Whatever purposes the authors of this Conference may have had in mind in bringing me here I hope the above questions were a part of it

Wright That is very interesting and I am glad that you brought it up because it seems to me that this represents a different approach than we have been discussing I have been feeling that in the last day or two—and we must constantly review our own endeavors and criticize ourselves—we have been stressing to too great a degree perhaps just one approach, one type of discipline in this field has been predominant Maybe it should have been predominant *I am glad however that another aspect, another facet* of this multi faceted field has been exhibited or polished up a bit

Are there other comments in reference to Dr Knisely's material? I would like to hear that explored a little bit further Dr Barker?

Barker I think that this is interesting and fundamental I have known about Dr Knisely's work for some time but I have never had an opportunity to hear him discuss it in this way These phenomena would seem to be fundamental in the pathogenesis of intravascular thrombosis possibly more so even than coagulation as we usually think of it I would like to ask Dr Knisely if he has repeated the experiments in animals that had been given heparin or dicumarol since there is a question as to whether the *anticoagulants may not have an additional inhibiting effect on the formation of thrombi besides their effect on coagulation as demon-* strated in test tubes

Knisely We have not yet put heparin or dicumarol into this exact type of experiment When a new piece of pathologic physiology is found the therapists have two choices one is to see if any of the active materials they have in their armamentarium work on this particular pathology the other one is to see if they can design some experiments and make some more observations I am sure as soon as this kind of experiment is known a lot of people will make tests with heparin and dicumarol

Barker You have the advantage of great familiarity with setting up the experiment whereas it would take some time *for me*

be necessary to identify everything that is in the great unknown mixture before we learn to recognize that certain experimental results may parallel some of the clinical problems This sort of thing looking at Y shaped vessels may be used to see what the rate of reaction will be the rate at which they leave Does that make any sense to the physical chemists or is it just a pipe dream?

Edsall I think that is the way you often proceed

Barker Dr Knisely s discussion recalls the observations of Hirschboeck on clot retraction in thrombosis and embolism Most of you are familiar with his tests done both in test tubes and by observing a drop of blood in oil Hirschboeck found that clot retraction was more rapid in patients with clinical venous thrombosis than in normal individuals and more rapid in patients with pulmonary embolism than in those with venous thrombosis without embolism We tried to repeat Hirschboeck s work but did not get the same results however perhaps we did not try hard enough or long enough

Wright Dr Quick I know that you have been interested in this

Quick The work of Dr Margory Zucker answers some of the questions Dr Barker raised She showed that platelet agglutination is definitely prevented by heparin and dicumarol I shall try to discuss this a little later

I enjoyed with profit the paper that was presented by Dr Flynn I was particularly happy because Dr Flynn has started using the one-stage method He and his associates will probably learn some of the advantages of the method Now let us begin with the statement that the one stage method does not measure prothrombin It measures prothrombin activity That to the pure chemist is probably annoying but to those of us who also deal with the bleeding patient prothrombin activity is very important Let me also remind you that the two-stage method when applied to blood and not to purified systems measures prothrombin activity and the proponents of the two stage method find it necessary to regulate and add the so-called accelerator globulin If the accelerator globulin is not taken care of the blood of a patient such as was described by Dr Owren would produce a most confused result with the two-stage method

In regard to the labile factor—and I prefer to use the term labile factor' because my work was one year earlier than Owren s

statistical distributions of the various patterns that physiology can be put into, plus the alterations during the death process When the material we were studying became too complex to grasp, I went up to Professor Robert R Bensley (an extremely wise man) and said 'There is too much here now We cannot keep track of all this", and the following is worth the record

The old professor said, Well now, the first thing to do is to find out what is normal living histology Once you know what the normal is you will then be able to recognize pathology by con trast Also you will know what you want to make therapeutic agents accomplish The purpose must be to make this pathology go back towards normal physiology"

There is the wisdom of a great man, at what would look like the end of a lifetime s experience At that time he was 65 years old but he has since been an active investigator for some 18 years We *got lost* in the beginnings of this When we started we thought we could find out what the normal was in six weeks and that was 18 years ago

Fremont-Smith May I throw in one comment without any criticism of Professor Bensley's remarks? Most often the effects are the opposite One does not usually discover pathology from the study of the normal but quite the reverse One learns the first approximation of the normal by studying pathology and I believe there are very few exceptions to this In fact almost all *scientific study is initiated by the observance of the difference,* the difference being an abnormality in a pattern or family Then the difference is observed more closely Soon there is a series of differences—really a new family Then a law is formulated to describe the new family One can almost trace the history of scientific discovery as a series of differences or pathologies Why does one start to work on a problem? Because something attracts the attention and this something is usually a difference

Knisely One movement in blood clotting has consisted of the isolation of *substances* involved in the clotting and thrombosing mechanisms, the determinations of the physical chemistry of the reactions and so on That was necessary and priceless and no adjectives that I can put together will overstate my admiration for it

Now we have many approaches possible when you come to face pathologic physiology, but I would like to suggest that it may not

one can postulate that another reaction occurs on storage, namely an alteration in component A The result is that because of the retention of the labile factor and the change in reactivity of component A a prothrombin time is obtained which is strikingly shorter than that of fresh normal oxalated plasma This same change in component A activity occurs however in 0 2 M citrated oxalated and amberlite plasmas If one adds to any of these plasmas a small quantity of fresh rabbit plasma treated with tricalcium phosphate which removes components A and B but leaves all of the labile factor and therefore serves as a highly potent source of the labile factor one observes a much shorter prothrombin time of the mixture than when a similar quantity of calcium phosphate rabbit plasma is added to fresh oxalated human plasma It is well to bear in mind, therefore that two reactions occur in stored plasma, the destruction of the labile factor and the potentiation of component A The latter change is usually masked by the lack of labile factor Perhaps these observations may have something in common with Seegers accelerator plasma and serum globulins Clearly the formation of thrombin is complex and may perhaps include several reactions

So much for that Only one more remark in regard to Dr Flynn s work I think the thing which counts (and that is very much in accord with Dr Knisely s work) is the speed with which the thrombin is made available Curiously if we do the prothrombin consumption test which consists of allowing 2 cc of blood to coagulate in a standard Wassermann test tube and then in 15 minutes determining the prothrombin time we find that the prothrombin time is probably 9 to 10 seconds in another 30 minutes it may be 15 and in another 30 minutes or an hour it may be 30 seconds I shall discuss this afternoon the significance of these findings in terms of hemophilia and in terms of thrombocytopenic purpura

Tocantins I would like to comment on the use of the term prothrombin time or prothrombin activity to designate the one stage method While we are discussing the propriety of using different terms—what we are actually doing when we add an excess of thromboplastin to either plasma or whole blood is accelerating the clotting time of the blood to a minimum and why not call it exactly that? Why not call it the *accelerated clotting time* of the plasma with the proviso that it bears a very close relationship to the amount of prothrombin in the plasma? But actually

—this labile factor I assayed for in the blood of various animals with the following results I found that man's contains very little, which is in agreement with the statement of Dr Seegers that man has very little of the accelerator globulin I found that the dog's contain ten times as much as the human Incidentally I found that the cow s contains about five times as much The rabbit's surprisingly, contains fifty times as much Those results obtained by a simple method using stored plasma as the assay medium gave me results which agree surprisingly well with those that were later reported by Dr Seegers The article that I refer to was published in the Journal of Laboratory and Clinical Medicine with my associate, Dr Stefanini [Vol 33 *819 (1948)*]

The stability of the labile factor is definitely associated with calcium If the calcium of human blood is removed with sodium oxalate, the plasma, in standing 24 hours at refrigerator temperature may show a prothrombin time of 15 seconds which indicates a reduction of 50 per cent prothrombin activity The same occurs when plasma obtained by passing blood through amberlite is stored This resin has no other action on blood but the removal of calcium Clearly then the removal of calcium causes a rather rapid disappearance of the labile factor Components A and B are not affected

With sodium citrate the results show interesting variations If one volume of 0 1 M sodium citrate is added to 9 volumes of human blood total incoagulability will be achieved, but the plasma thus obtained will in 24 hours show a distinct shortening of the prothrombin time It may be as low as 9 seconds which might easily be erroneously interpreted as hyperprothrombinemia If instead of 0 1 M sodium citrate 0 2 M is added to 9 volumes of blood the resulting plasma on 24 hour storage shows the usual drop of prothrombin activity Apparently the higher concentration of sodium citrate removes sufficient ionized calcium to cause the labile factor to deteriorate Although 0 1 M sodium citrate can cause complete incoagulability it does not remove all the ionized calcium and, therefore the labile factor remains stable The reason 0 1 M sodium citrate acts as an anticoagulant is that it combines with prothrombin and actually acts as an antiprothrombin [*J Gen Physiol* 32 *191 (1948)*]

How can the abnormally short prothrombin time in 0 1 M sodium citrate plasma on storage be explained? In the first place the labile factor remains undiminished and in the second place

that question satisfactorily answered? If it was not put the question again and give Dr Quick another chance

Seegers I believe I asked Dr Quick the question yesterday as to what he meant by the term 'prothrombin complex'

Fremont Smith Have you been answered satisfactorily?

Seegers Not to my knowledge

Quick May I postpone answering that question till this after noon?

Best Did not Nolf describe what you call the labile factor?

Quick Nolf has very little claim to being first to identify the labile factor He has made many suppositions and one of these is that the platelet is not essential—that the plasma contains all the elements necessary for coagulation This is fundamentally errone ous and to a large extent undermines his whole theory

Seegers I would like to point out that some of the remarks that Quick has made do not agree with the work of his own student Honorato This work concerns calcium and has been recently published in the Journal of the Society for Experimental Medicine Also I would like to point out that the use of prothrombin complex was at one time mixed up with labile factor

Quick The labile factor is still part of the prothrombin com plex

Seegers It was necessary for us to point out that it was not the prothrombin the substance which becomes thrombin which disappears in storage plasma If labile factor and Ac globulin are one and the same thing then labile factor is scarcely labile It is labile under one condition namely in oxalated human plasma Even Dr Quick himself did work on oxalated plasma and made predictions to the clinician as to what would happen in citrated plasma I think that those things need to be brought out and also I believe it is as important to describe the mechanics and mechanisms of how these things work as it is to invent new words

I would like to point out that before we said anything in the literature about Ac globulin we had electrophoresis curves of con centrates of Ac globulin Also we have pointed out in detail its relationship to serum Ac globulin I might say something about the mechanics of its activation of prothrombin Such studies I think are far above the recording of 14 second clotting times and the comparing of them with 9 and 10 second clotting times where you have many variables including the variable of species—

the descriptive term *should be the accelerated clotting time of the* plasma or of the blood, rather than the prothrombin time

Flynn I would like to tell Dr Quick that I appreciate his interpretation of the accelerated clotting time observed in stored citrated blood About a year and a half ago, we started, as a side issue to see how many variables were involved in the prothrombin determination on aged human blood We observed the hypercoagulability effect (one-stage method) that occurs after a few days of storage However, we were unable to pin down the exact mechanism *causing this apparent anomaly Dr Quick s* interpretation *is* an interesting one

Quick In answer to Dr Tocantins' suggestion about the accelerated clotting time a name itself means little The term actually was suggested by the English workers I don't believe you can ever get people to use it because the term prothrombin time, is now firmly established all over the world and when a term once has been accepted there is little chance of ever making people change The clinical pathologists have deleted the word "platelet from their list of names and they say, *' We shall name these structures* thrombocytes I will venture to say that they will never succeed in abolishing the term platelet"

Fremont Smith Dr Quick has touched on the problem of terminology Our attachment to words is exceedingly interesting One time in the World Court at The Hague Manley Hudson was asked how it was possible for him to speak on controversial topics without arousing antagonism He replied that it was very simple because he learned early in his career that people's emotions are attached not to ideas but to the words associated with certain ideas hence, when he wanted to talk on controversial topics without arousing antagonism he was careful to use a vocabulary not ordinarily associated with the group In this way he had no difficulty whatsoever in introducing the idea but once let him use a familiar vocabulary all the blind spots were activated

However, this is really not prefatory to another remark I want to make One of the purposes indeed one of the advantages of these group discussions is that one can come back at a man who makes a statement and ask him to elaborate If he fails to do so one can keep interrogat*ing him until he either does so or* admits his position to be untenable

I remember yesterday Dr Seegers asked Dr Quick a question and a little later asked him the same question Dr Seegers was

THE PRESENT STATUS OF THE PLATELETS IN COAGULATION

ARMAND J QUICK

Department of Biochemistry Marquette University School of Medicine

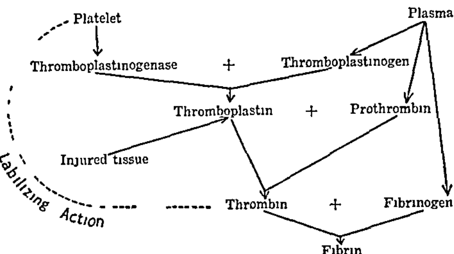
In my monograph on hemorrhagic diseases I stated that thrombin must be regarded as the central substance in the mechanism of coagulation and that the platelet is the most fundamental factor in hemostasis since it appears to participate in all the known mechanisms utilized by the body to control the loss of blood The correctness of these two statements has not only been amply substantiated by the important developments that have occurred since the monograph came off the press but now a definite relationship between thrombin and the platelet can be offered

At least three distinct functions of the platelets in hemostasis are known (1) participation in the formation of thrombin (2) formation of white thrombi to serve as plugs for stanching and (3) vasoconstriction Strangely enough their first function has not been clearly defined and has remained controversial Three quarters of a century ago both Bizzozero and Hayem showed that coagulation ensues where platelets disintegrate Yet for many years particularly in England the very existence of platelets was questioned and even present-day investigators including Nolf [*Medicine* 17 *381 (1938)*] Lozner and Taylor [*J Clin Inves* 21 *241 (1942)*] Lenggenhager [*Schweiz Med Wchnschr* 76 *411 (1942)*] Ferguson [*Science* 97 *319 (1943)*] and others postulate that they are not required for coagulation

By means of a new tool a silicone compound which supplies a surface that preserves platelets Jaques et al [*Canadian Med Assn J* 55 *29 (1946)*] showed that coagulation was markedly retarded when platelets were removed This finding has been confirmed by Brinkhous [*Federation Proc* 6 *389 (1947)*] and by me *(Federation Proc* 6 *284 (1947)*] and recently Seegers and co workers [*Blood* 3 *656 (1948)*] showed that with complete removal of platelets no coagulation takes place Equally important was my observation that the removal of platelets resulted in a markedly defective conversion of prothrombin to thrombin [*Am J Med Sci* 214 *272 (1947)*] By means of my prothrombin time determination and by my new procedure the pro-

i e, rabbit plasma and stored oxalated plasma, etc I think all of those things have an important bearing on getting at what is really going on I would also like to point out that Dr Owren added to all this the clear demonstration that a patient can have a deficiency of factor V and that he is in very real trouble with such a deficiency

The position of the platelet in the coagulation mechanism can be simply presented by the following scheme



Instead of two reactions as given for the classical theory, there are at least three distinct steps

(1) Thromboplastinogen $\xrightarrow{\text{platelet enzyme}}$ Thromboplastin

(2) Prothrombin complex + Thromboplastin = Thrombin

(3) Fibrinogen $\xrightarrow{\text{thrombin}}$ Fibrin

It is to be noted that the first and third reactions are enzymatic whereas the second appears definitely to be stoichiometric Actually it is very likely that the second step is but an overall expression of several reactions since the prothrombin complex contains at least three distinct factors

According to this scheme all the commonly known coagulation defects other than afibrinogenemia and heparemia can be grouped under two general headings (1) normal prothrombin time and (2) prolonged prothrombin time In the first class belong the two important diseases thrombocytopenic purpura and hemophilia Both have in common a lack of available thromboplastin which results in a poor consumption of prothrombin and

thrombin consumption test I was able to demonstrate that platelets contain no significant amount of thromboplastin as was hitherto believed, but that they supply an activator of thromboplastin I postulated that the latter exists in plasma as inactive thromboplastinogen The action of platelets, as measured by the prothrombin consumption test, is typical of an enzymatic reaction as shown in Figure 25, in which the speed of the reaction is plotted against the number of platelets Recently Ware Fahey and Seegers [*Am J Physiol* 154 *140 (1948)*] have confirmed my finding that platelets do not contain a significant amount of thromboplastin but supply an activator Their explanation of the action of this activator which is not in accordance with mine I leave for Dr Seegers to discuss

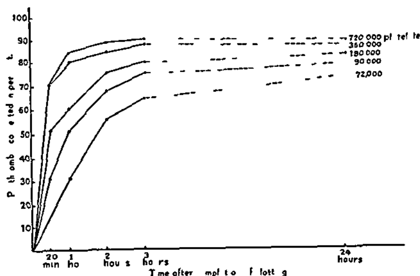


FIG 25

When the prothrombin time is prolonged the defect in coagulation must be sought in the prothrombin complex From the known types of congenital hypoprothrombinemia, one must conclude that there are at least three factors (components A B and Labile Factor) in addition to bound calcium When the prothrombin time is normal all these factors can be assumed to be qualitatively and quantitatively normal This is important in understanding the true nature of the chain reaction in coagulation

It has long been known that agents which inhibit coagulation also retard or prevent platelet disintegration Among such agents may be mentioned oxalates citrates heparin amberlite and hirudin In addition platelet stability is also noted in hemophilia and in marked hypoprothrombinemia induced by dicumarol What is the common denominator in all these? Clearly it is the lack of *thrombin formation From this I have postulated that thrombin* has a labilizing action on platelets resulting in a faster disintegration of these cells and consequently more activation of thromboplastin This in turn produces more thrombin which immediately labilizes additional platelets thus setting off a chain reaction This explains why a minute quantity of thromboplastin from tissue inadvertently gaining entrance in the collection of a specimen of blood exerts a seemingly disproportionate effect even when silicone coated glassware is employed A minute amount of free thromboplastin immediately produces an equivalent quantity of thrombin which sets off the chain reaction In hemophilia even a large amount of thromboplastin will not start this autocatalytic reaction because of an essential break in the chain namely the lack of thromboplastinogen The effect of adding thromboplastin to hemophilic blood is that of a stoichiometric reaction Since the prothrombin complex is normal in hemophilia it follows that the basic chain reaction is not mediated through the prothrombin complex In fact the prothrombin activity must be tremendously depressed before platelet disintegration is inhibited When thrombin is very drastically depressed the platelets are stable even when blood is kept in a glass container In a carefully collected sample of blood free of extraneous thromboplastin the platelets remain intact if the container is coated with an agent such as the silicone dri film

Fibrinogen apparently does not participate directly in the labilizing action of thrombin on platelets for Pinninger and Prunty

therefore to an inadequate production of thrombin In thrombocytopenic purpura, it is the prothrombin consumption which is the true index of impaired coagulability [Quick Shanberge and Stefanini, *J Lab Clin Med*, 33 *1479 (1948)*] It may be drastically reduced as shown in Table 23 The coagulation test is normal and therefore in the past this has been erroneously interpreted to mean that no impairment of coagulability occurs in this disease In hemophilia the platelets are normal on adding washed hemophilic platelets to normal deplateletized plasma, normal coagulation results It appears fairly certain that hemophilic blood lacks thromboplastinogen

TABLE 23
THROMBOCYTOPENIC PURPURA
PATIENT BOY AGE 6 YEARS

Date	Platelet Count	Prothrombin Consumption	Beginning of Clot Retraction	Bleeding Time	Clinical Condition
June 4	26,000	13 sec	27 min		Marked Bruising
June 8	25 000	11 sec	29 min	10 min	Marked Bruising
June 12	49,000	13 sec	18 min	5½ min	Marked Bruising
June 17	13,000	13 sec	15 min	16 min	Marked Bruising
June 24	15,000	11 sec		12 min *	Marked Bruising
July 1	29,000	14 sec	14 min	9½ min	Marked Bruising
July 14	98 000	20 sec	11 min	2½ min	No Bruising

* Felic Acid 20 mg daily

Even an extract of ground platelets will not change the coagulability of hemophilic blood, since it is not a lack of platelet enzyme but a lack of the thromboplastin precursor that is the basic defect One rare condition may also be included under the first heading namely the acquired hemophilia like diathesis In this disease evidence has been obtained showing that the blood contains an antagonist to the platelet enzyme [Quick and Stefanini *Proc Soc Exp Biol & Med 47 111 (1948)*]

From the material which I have presented it appears evident that the statement from my monograph, By using the platelet as the common denominator the organism is able to coordinate all the mechanisms of hemostasis epitomizes the whole subject The essential steps of hemostasis can be clearly discerned When a blood vessel becomes injured it contracts promptly presumably by a sympathetic nerve reflex This slows circulation and allows platelets to adhere to the injured vessel wall Very likely a little thromboplastin enters the blood from the traumatized vessel Enough thrombin is formed to labilize the platelets in the localized area This results in a platelet thrombus which is adherent to the injured vessel As platelets disintegrate in this thrombus thromboplastinogen is activated and thrombin is formed

Fibrin consequently is formed at the point where thrombin originates which is the platelet clump The fibrin thereby becomes anchored in the platelet residue that is strongly adherent to the vessel wall Furthermore the disintegrating platelets liberate a vasoconstricting agent which is undoubtedly of great importance Thus the first step in hemostasis is the formation of platelet thrombus the second is the vasoconstriction due to platelet lysis By these two means surprisingly good hemostasis is achieved as is attested by the fact that the afibrinogenemic subject has a relatively mild hemorrhagic condition Fibrin formation is distinctly a third line defense It assures a permanent and secure closure of the vessel From an evolutionary point of view it is a late development that becomes necessary only after the blood pressure becomes increased to such a degree that the more primitive measures no longer afford adequate protection

In summary it is the platelet that is the central coordinating factor in hemostasis Its role in the production of white thrombi in the formation of thrombin and in maintaining vasoconstriction supplies three pillars on which the hemostatic mechanism is built The activity of the platelet is inseparably linked with thrombin in fact so much so that one can consider the labilizing action of thrombin on platelets a far more important function than its property of converting fibrinogen to fibrin

DISCUSSION

Wright I will ask Dr Brinkhous to open this discussion

Brinkhous Dr Quick and myself appear to be in agreement

[*Brit J Exp Path* 27, *200 (1946)*] found that normal platelet disintegration occurred in afibrinogenemic blood

Clot retraction logically follows as the next topic to be discussed This phenomenon is dependent upon intact platelets and thrombin To Bordet and Delange, and Le Sourd and Pagniez we owe the knowledge that only intact platelets can cause clot retraction Tocantins [*Am J Physiol* 114 *709 (1936)*] pointed out that platelets become adherent to the shafts of fibrin and that as the platelet groups fuse, they cause a bending and twisting of the fibrin strands with resultant retraction

During the past year, we have studied the problem of clot retraction and have come to the following conclusions (1) the number of platelets determines both the speed and the degree of clot retraction (2) the same chain reaction occurring in coagulation is operative in clot retraction i e the activity of platelets depends on their labilization by thrombin By adding thrombin to hemophilic plasma rich in platelets, rapid retraction results Likewise, addition of thrombin to oxalated plasma brings about prompt retraction showing that calcium is not required The fact *that clot retraction occurs rapidly in hemophilic and in oxalated* plasma following the addition of thrombin is further evidence that the latter agent labilizes platelets

The second function of platelets in hemostasis namely, their clumping to form plugs to seal small injured vessels, is even now not fully appreciated though such descriptive term as *clau hemostatitique* was applied to white thrombi *forty years ago* In a recent work which is outstanding Zucker [*Am J Physiol* 148 *275 (1947)*] has clearly shown by direct microscopic observation that platelet agglutination at the site of the injured vessel plays a major role in controlling bleeding Intimately associated with the mechanical function is the vasoconstriction action which is brought about by the liberation from the disintegrating platelets of an agent which contracts injured as well as normal vessels Zucker found that the formation of platelet plugs was inhibited by heparin and by dicumarol induced hypoprothrombinemia and that likewise no platelet clumps were found in experimental thrombocytopenic purpura

The nature of the vasoconstricting agent liberated by platelets is still entirely unknown Since the platelets have also been found to contain histamine, one can see that any simple explana is unlikely to be adequate

quantitative basis using the technique previously described for determination of prothrombin utilization [K M Brinkhous *Am J Med Sci* 198 *509 (1939)*] These experiments have been done on both human and dog blood Platelet numbers have been regulated by one of three methods (1) Centrifugation of blood for varying periods of time By controlling the time and centrifugal force the desired number of platelets can be obtained (2) By mixing platelet rich and platelet poor plasmas in varying proportions (3) The addition of washed concentrated suspensions of platelets to platelet poor plasma The rate of prothrombin consumption in plasmas containing reduced numbers of platelets was compared with normal whole blood In general the same results were obtained in both species and regardless of the method used for adjusting platelet concentration The results can be summarized as follows (1) with extremely low platelet levels no decrease in prothrombin is detectable in the course of the experiment (2) with platelet levels one third to one fourth of the normal values or less a significant delay in prothrombin utilization occurs and (3) with higher platelet levels as much prothrombin is utilized in a given time as with a full complement of platelets These results appear to be at variance from the data presented by Dr Quick This discrepancy probably is the result of the method used for detecting residual prothrombin in serum We used the two-stage method

The mechanism by which platelets make thromboplastin available is debatable Howell Lenggenhager and others have postulated for many years that a plasma thromboplastin or thromboplastin precursor exists in plasma Dr Ferguson has mentioned repeatedly that a thromboplastic enzyme is present in plasma which in some way mobilizes thromboplastic material Data now available clearly indicate that an interaction between platelets and plasma is necessary for the inception of clotting Just what is involved in this reaction must await further study Dr Quick has stated one possibility If I understand correctly his hypothesis is based on two bits of data—analyses of platelets for thromboplastin which gave essentially negative results and rates of disappearances of prothrombin from plasma with different platelet levels In my opinion the data are rather tenuous We have suggested another interpretation of the available facts—thromboplastin exists preformed in platelets and the plasma platelet interaction results in thrombocytolysis and thromboplastin liberation Many other interpretations undoubtedly are possible Macfarlane for one

regarding the functional importance of platelets in the fibrin clotting mechanism I would like to refer to some old data which will supplement the historical background briefly outlined by Dr Quick About 10 years ago, incidental to a study of the hemophilic clotting defect *a relationship between the rate of disappearance of prothrombin from clotting blood and the platelet content* was noted [K M Brinkhous, *Am J Med Sci* 198 *509 (1939)*] If normal human whole blood is centrifugalized at high speed immediately after its withdrawal from the vein the rate of prothrombin utilization is much less than in the whole blood not subjected to centrifugation In one hour after the onset of clotting as much prothrombin disappears from the whole blood with its full complement of formed elements as occurs in four hours in the centrifugalized specimen This difference was attributed to the presence of abundant platelets and their disintegration products in clotting whole blood in contrast to the smaller number of platelets in the centrifugalized specimen It was postulated that this difference would be even more pronounced if one obtained plasma completely free of platelets Unpublished experiments done somewhat later in 1939 and 1940 failed to accomplish this goal largely in retrospect, because of technical difficulties associated with using equipment having a wettable surface It was not until Jaques and coworkers introduced silicone some six years later that another test of this hypothesis could be made The results of experiments of this type are now well known [K M Brinkhous *Proc Soc Exp Biol and Med* 66 *117 (1947)*] We reported our data on this subject in part at the first conference here last year Since that time somewhat similar data have been obtained with dog blood by T B Patton A G Ware and W H Seegers [*Blood* 3 *656 (1948)*] In the best preparations no prothrombin is utilized in platelet free preparations when the mixtures are placed in ordinary glassware It is relatively simple to obtain platelet poor normal plasma in which prothrombin consumption is minimal if the test is made in silicone coated glass tubes In our best preparation no prothrombin was utilized at all in ordinary glass Return of platelets to the system results in prompt clotting and a normal rate of prothrombin utilization

Qualitatively then there is an obvious relationship between the platelets and the rate at which prothrombin is converted into thrombin J A Buckwalter, W B Blythe and I (unpublished data) have been interested in placing this relationship on a

quantitative basis using the technique previously described for determination of prothrombin utilization [K M Brinkhous *Am J Med Sci* 198 *509 (1939)*] These experiments have been done on both human and dog blood Platelet numbers have been regulated by one of three methods (1) Centrifugation of blood for varying periods of time By controlling the time and centrifugal force the desired number of platelets can be obtained (2) By mixing platelet rich and platelet poor plasmas in varying proportions (3) The addition of washed concentrated suspensions of platelets to platelet-poor plasma The rate of prothrombin consumption in plasmas containing reduced numbers of platelets was compared with normal whole blood In general the same results were obtained in both species and regardless of the method used for adjusting platelet concentration The results can be summarized as follows (1) with extremely low platelet levels no decrease in prothrombin is detectable in the course of the experiment (2) with platelet levels one-third to one-fourth of the normal values or less a significant delay in prothrombin utilization occurs and (3) with higher platelet levels as much prothrombin is utilized in a given time as with a full complement of platelets These results appear to be at variance from the data presented by Dr Quick This discrepancy probably is the result of the method used for detecting residual prothrombin in serum We used the two-stage method

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has suggested that a thromboplastic factor in platelets is potentiated by a plasma enzyme Obviously more data are needed to decide the nature of the plasma platelet reaction It may be that two separate reactions are involved, one having to do with platelet breakdown and another with the potentiation of thromboplastin

In addition platelets furnish accessory clotting factors not related to thromboplastin For example (1) the platelet accelerator of Mann, Hurn and Magath [*Proc Soc Exp Biol and Med*, 66 *33 (1947)*] and (2) the platelet factor that acts similarly to acacia on the thrombin fibrinogen reaction as described by A G Ware, J L Fahey and W H Seegers [*Am J Physiol* 154, *140 (1948)*] Tocantins for many years has emphasized the importance of platelets in determining the physical character of the clot and syneresis Apparently then, platelets are disrupted after blood is drawn and platelet protoplasm diffuses throughout the plasma adding a number of coagulant factors The need for careful collection of blood to prevent platelet rupture is imperative, then in any study of coagulant factors present in plasma Otherwise a factor may be considered of plasma origin whereas in reality it originated in the platelets, or the plasma factor may have been modified by interaction with liberated platelet material

Finally, we have been less successful in preserving platelets if oxalate is used as an anticoagulant instead of citrate Perhaps the calcium oxalate precipitate provides a large surface conducive to viscous metamorphosis of platelets

Ferguson In our current work concerning the effects of platelet material in isolated test systems of purified prothrombin converting into thrombin with various activators etc B L Travis Jessica H Lewis and I have a number of experimental data Although our analyses are not yet complete I should like to show you some typical data because of their bearing on the ideas presented by Dr Quick and Dr Brinkhous Table 24 illustrates some of these data

TABLE 4 — POTENTIATION OF PLATELET THROMBOPLASTIN

Clotting times of sample of thrombic mixture (T) added to test fibrinogen after incubation periods stated T mixtures contain prothrombin and various activators and inhibitors cited with borate buffer to constant volume pH=7 75 temp =25 C

T	ACTIVATORS	INHIBITORS	INCUBATION PERIOD 5 min	10 min	20 min	40 min	1 hr	2 hr	3 hr	6 hr	48 hr
1	Ca tpln Ac G	—	27	21	19	19	19	19			
2	Ca, tpln Ac G + platelets	—	31	22	19	19	19	19			
3	Ca Ac G	—	∞	∞	∞	∞	∞	∞*			
4	Ca Ac G + platelets	—	720	540	270	100	65	38	—	28	
5	Ca Ac G trypsin	—	182	140	95	58	51	—	52		
6	Ca Ac G trypsin + platelets	—	27	19½	16	17	17½	—	20½		
7		antilysin	710	490	205	103	71				
8	"	pancr inhib	660	240	175	113	96				
9	Ca Ac G lysin	—	390	380	368	335	320	—	250	200	—
10	Ca Ac G lysin + platelets	—	77	57	39	37	37				
11		antilysin	73	46	37	36*	35				
12	"	pancr inhib	105	65	57	53	52	—	51	—	50
13	Ca Ac G thrombin + platelets	—	130	76	51	42	42½				
†14	Ca Ac G thrombin	—	220	220	200	160	135				
†15	Ca thrombin	—	555	490	480	670	720				
†16	Ca + Thrombin (no prothrombin)	—	9¼ m	11 m	13½ m	17¼ m	20 m				
†17	Ca + Lysin (no prothrombin)	—	No clot owing to Fibrinogenolysis 2 hrs								
†18		antilysin									

* ∞ no clot in ½ hr

† Tests 14 18 were made with *prothrombin-free* fibrinogen $BaSO_4$ adsorbed BF

Method As described previously clotting times for a test solution of fibrinogen (BF) are noted following addition of a measured sample of comparable thrombic mixtures (T), incubated for varying periods after mixing the prothrombin (Pro) with optimal $CaCl_2$ and the other activators (and inhibitors) cited

Reagents 1 (BF) bovine fibrinogen (Armour s), 2 (Thr) Seegers highly purified bovine thrombin, 3 *(Pro) Seegers purified* bovine prothrombin, 4 (Ac G) Seegers' bovine serum accelerator globulin", containing a little prothrombin 5 (tpln) dog brain thromboplastin 6 (plat) well washed dog platelet suspension (see below), 7 (tryp) Kunitz's crystalline pancreatic trypsin, 8 (lysin) Loomis bovine plasma protease (fibrinolysin) 9 (antilysin) Loomis' bovine plasma protease inhibitor (antifibrinolysin), 10 (pancr inhib) Kazal s crystalline pancreatic trypsin inhibitor, 11 (AHG) Harvard human plasma Fraction I (antihemophilic globulin")

Results (See Table 24)

The conversion of prothrombin to thrombin is typically complete in 15 20 min with the weak (1 25,000) prothrombin (aged), *activated in the presence of added Ac G (1 25 000 final) and optimal* $CaCl_2$ and thromboplastin (T 1) The extra addition of platelets has no significant effect (T 2) Ca Ac G (alone) do not activate the prothrombin (T 3) ∞ = no clots in ½ hr but the addition of platelets (T 4) provides sufficient 'thromboplastic' factor for a significant though slow and incomplete thrombin formation Crystalline trypsin has a considerable 'thromboplastic effect (T, 5) Trypsin *plus* platelets affords an excellent potentiation of thromboplastic effects (T 6), slightly better if anything than T 1 In T, 7 T 8 the trypsin potentiation of the platelet thromboplastic action is abolished by the antienzyme preparations from plasma (antilysin) and pancreas (pancr inhib) respectively the residual thromboplastic action being very similar to that originally obtained (T 4) with the platelets alone

The lysin preparation seems to resemble trypsin in definitely potentiating the thromboplastic action of platelets (T 10) Unlike the trypsin experiments however those with the lysin show no significant reduction of the potentiating effect by the anti proteases (T, 11 T 12) The lysin without platelets (T 9) has a very weak but not negligible thromboplastic' effect Owing to fibrinogenolysis (T, 17), it is difficult to be sure that the enzyme

preparation is completely free from all traces of thrombin In the presence of optimal calcium which has a markedly accelerating effect on the clotting of fibrinogen with very weak thrombins we have observed some clotting in a couple of hours (T 18) of prothrombin free fibrinogen ($BaSO_4$ adsorbed BF) by lysin *plus* antilysin proteolysis being inhibited and the antilysin alone having no such effect. It is difficult to feel that such negligible traces of thrombin activity can be significant but we did feel the need for investigating the effects of weak thrombins on our platelet-containing test systems A highly purified thrombin (one of Dr Seegers best preparations) was diluted until (with Ca) it gave a 9½ min clotting time deteriorating (as usual) to a 20 min c t in the 1 hr incubation period (T 16) This weak thrombin in the platelet experiment (T 13) showed a significant thromboplastic potentiation resembling that observed with the enzyme preparations Some additional control tests made on another occasion with a prothrombin free fibrinogen showed a similar thrombin to have no activating effect on prothrombin in the presence of Ca (T 15) and very little when Ac G was also added (T 14)

We have other tests attempting to combine the effects of platelets and antihemophilic globulin on prothrombin activation Owing to the interfering fibrinogen in the AHG preparation and the risk of losing the antihemophilic factor by heating or precipitation techniques we were forced to defibrinate the AHG with a little thrombin Since there is no significant amount of antithrombin in the AHG solution there was always a trace of thrombin left after the defibrination We did find this defibrinated AHG to give marked potentiation of the platelet thromboplastic effect but in view of experiments similar to the above where comparable amounts of thrombin were also able to cause this effect no part in this action could with any certainty be attributed to the antihemophilic factor We had previously shown [*Proc Soc Exp Biol and Med* 67 *228 (1948)*] that AHG was quite devoid of any accelerator effect in the activation of Ac-G poor prothrombin by optimal calcium and brain thromboplastin The somewhat inhibitory effects which we obtained instead could have been due to some thrombin removal by the traces of clot that did form in the T mixtures in these experiments

We wish to pursue these studies further before we can feel fully satisfied that traces of thrombin can account for potentiation

of the thromboplastic action of platelets, but our tentative con clusion is along these lines and this could be an important thing under natural clotting conditions

The point we wish strongly to present, in relevance to the claims made this afternoon by Dr Quick, is that many agents, apparently unrelated (unless we are right about the traces of thrombin in many of them) can be shown experimentally to assist platelets in the conversion of prothrombin to thrombin Dr Quick will need to adduce much more positive evidence before we can accept his theoretical suggestion of a new plasma factor or "throm boplastinogen' If we stick to the facts, the theories will take care of themselves

In comparing our platelet experiments with those of Ware Fahey, and Seegers [*Am J Physiol*, 154, *140 (1948)*] I should like to note that we find little or no evidence of Ac G like factor in our platelet suspensions I might detail our method, as it raises one or two interesting technical points

Preparation of Platelet Suspension Dog blood is collected by veni puncture into a 50 cc syringe containing 1/8 vol of isotonic (3 5 per cent) trisodium citrate The blood is immediately centrifuged in lusteroid tubes, first at 1 000 r p m for 10 min in the refrigerated centrifuge (2° 5° C) and the pipetted off supernatant plasma spun again at the same speed until a white cell layer begins to sediment The plasma is once more removed to another lusteroid tube and now centrifuged at 5,000 r p m for 10 min (cold) The supernatant is poured off and the platelet sediment resuspended in citrated (0 4%) saline To ensure thorough resuspension for adequate washing it is found necessary to allow this to warm up to room temperature for ½ hr, with vigorous stirring at intervals The platelets are again thrown down at 5 000 r p m and the wash ing, etc repeated a second and third time No visible red cells but usually a very faint pink tinge appear in the final sediment Hemo lysis incidentally, is completely absent in these plasmas even after a week at icebox temperatures The final platelet suspension is made up in 5 cc of saline (0 9 per cent NaCl) Wright stained films show the platelets to be discrete and apparently well preserved Only an occasional leukocyte and very rarely an erythrocyte are to be seen We believe that the care in the resuspension during the washings is important in order to get rid of plasma contaminants

We are at present just beginning to try the siliconed tube methods I do believe that the new silicone technique has reopened

the question of the role of platelets in blood coagulation I regard as most significant the success of Dr Brinkhous in the difficult experiment of securing platelet free plasma which fails to clot not only in siliconed tubes but even when transferred to glass I must still raise the question however whether there is not the possibility that the non wettable (e g siliconed) surface in addition to preserving the platelets may also modify certain colloidal systems in the plasma itself I recall the interesting experiment of Lampert and Ott (1934) in which blood was allowed to clot in a large glass tube with a longitudinal strip of paraffin down half the inside Clot retraction eventually occurs with the fibrin drawing away from the glass and remaining in contact with the paraffin My suggestion is that the wettable surface favors the mobilization of a serum protease which digests away the fibrin attachments in order to permit syneresis The well known significance of platelets in relation to clot retraction could perhaps be brought into line with this proteolytic theory of clot retraction It is certainly true as the older workers found and we have repeatedly confirmed that washed platelet suspensions do not cause clot retraction in purified thrombin fibrin mixtures Neither do such clots undergo fibrinolysis with any regularity We have not yet been able to show definitely that platelets activate prolysin (the plasma protease precursor) It does seem reasonable however to have in mind a system in which a plasma factor and a platelet factor interact to give clot retraction and proteolytic effects Whether such a system might also be an essential factor in thrombin formation is quite a question It is along these lines that we are pursuing our present investigations

Conley Dr Quick has implied as a number of other people have that normal plasma rendered platelet free will not clot However Dr Quick told me he had not actually achieved such an experimental result Dr Brinkhous was able to do that using citrated blood but I don t believe anyone aside from Fuchs claims to have obtained a spontaneously incoagulable human plasma without the use of anticoagulants hence it is presumptous of Dr Quick to say that plasma does not clot in the absence of platelets It may be so if the technique could be improved to remove the platelets more adequately

Perhaps that brings up an important point because Dr Flynn questioned the possibility of removing all the platelets from plasma by centrifugation However most of the platelets can be removed

quite rapidly by only moderate centrifugation If the remainder cannot it must be that the platelets have a very large range of density Some must be very much less dense than others I would like to know if there is any evidence of variation in density

We have, on a very great many occasions attempted to obtain a platelet-free plasma that did not clot We never succeeded in doing so and that led to the conclusion that perhaps platelets are not essential to clotting The results we obtained are amazingly similar to those Dr Quick obtained Plasmas which are platelet poor show very low prothrombin consumption and the amount of prothrombin converted to thrombin is directly related to the platelet concentration

We found one other very interesting thing which concerned platelets and it is that the action of heparin on blood is directly related to the platelet concentration The more platelets there are, the more heparin is required to render the blood incoagulable When the platelet count is low, an amazingly low cencentration of heparin will suffice to inhibit coagulation completely The order of magnitude of the change is about twenty fold, that is a concentration of heparin which will inhibit the clotting of essentially platelet free plasma is twenty times less than the concentration of heparin necessary to inhibit the clotting of blood containing a normal number of platelets I think the existence of plasma thromboplastic factors can hardly be denied if one tries to explain hemophilia and other hemophilia-like diseases which Dr Quick and I have studied

One other interesting study which we carried out and Dr Quick mentioned also is the relation of platelet concentration to clot retraction in silicone tubes We found as he did, that the correlation between the degree of retraction and platelet count was a very striking one Clot retraction did occur down to 20 000 platelets

Flynn The point I made about platelets was as follows plasma subjected to 1900 g for 180 minutes still contains platelets That this is so is demonstrated by the fact that if some of the supernatant plasma is put in the multispeed rotor at 31 000 g the sediment obtained will contain platelets I reemphasize Dr Tocantins' comment about using the term ' platelet-poor plasma instead of platelet-free plasma

Dr Conley's observation that the amount of heparin needed to produce incoagulability of blood is less for platelet poor plasma

probably means that the two effects merely supplement each other obviously blood rendered partially incoagulable by removal of platelets will require less heparin to produce total incoagulability

Seegers I think there is another point that might be added to that. I can appreciate the efforts that Dr Conley has gone through trying to get incoagulable plasma I can also see however where even with the finest of silicone technique one might still be confronted with the possibility that long before the plasma was drawn it already had some platelet decomposition products in it. I think the burden of proof is still upon the advocates of thromboplastinogen I would like to see a concentrate of it That might help us tremendously

Conley In regard to Dr Seegers point there is evidence which we feel is convincing that we have not activated platelets or released active thromboplastin in our centrifuging process Our plasmas clot in glass but not in silicone or if they do clotting times are very much prolonged So the glass surface does something to the platelet-free plasma It may do it to a platelet fraction I am sure I don t know At least we are not able to detect platelets in this plasma by direct microscopic examination I think the point that you don t believe a factor exists until you see it in a test tube can be carried too far The recent studies on Vitamin B^{12} for example illustrate this point You would have great difficulty in detecting B^{12} in blood One microgram in the total body fluid is all that is needed to produce a remarkable effect, so I do not believe it is necessary to wait until a preparation is in a test tube to be certain of its presence The cases which I mentioned have to be explained Situations that have occurred are facts and some explanations have to be given for their occurrence If you have an alternative explanation I would be delighted to hear it.

Seegers I would do a two-stage prothrombin and a two-stage Ac globulin analysis

Conley How would that inhibit coagulation of normal blood? These patients have a clotting inhibitor that tremendously prolongs almost completely inhibits clotting if enough of the plasma of these patients is added to normal blood

Seegers I just answered the question You wanted some suggestions as to what might be done and I gave them

Flynn This is a minor point but I think Dr Best might be interested About a year and a half ago we obtained some hirudin

from England We thought it would probably turn out to be heparin but we were hoping it might be different Physiologically, it acted just like heparin so we lost interest I might say, however, that we did not check the apparent identity of hirudin with heparin by qualitative chemistry

Quick I would like to make just two comments particularly in reference to Dr Brinkhous' discussion I want to emphasize that Brinkhous did some of the early work on the slow conversion of prothrombin Again it was a difference of interpretation He stressed the slow conversion of prothrombin whereas in my work I came to the conclusion that prothrombin was not converted The supply of thromboplastinogen was soon exhausted and therefore the prothrombin was not converted Brinkhous work was done in 1940 was it not?

Brinkhous It was done in 1938 and published in 1939

Quick That work is well worth reviewing

The other comment that I want to make is in regard to his finding that oxalate is poor for the preservation of platelets I agree and I think the explanation is that sodium oxalate acts relatively slowly whereas sodium citrate acts immediately During the long period required for sodium oxalate to make the blood incoaguable, enough thrombin is formed to labilize platelets and thus cause their disintegration May I refer you to a study which Dr Stefanini and I carried out [*J Gen Physiol* 32 *191 (1948)*]

I might also make this remark concerning foreign surfaces I happened to get my centrifuge too cold one time and tiny spicules of ice formed in the plasma The whole experiment was ruined because the ice acted as a foreign body for the platelets and caused their agglutination and disintegration

Seegers I would like to ask Dr Quick a question Do you regard the platelets as containing thromboplastin?

Quick No, I think that the amount of thromboplastin that the platelets contain is so small that it does not play a vital function Probably all cells contain some thromboplastin including the red cell and that is why I think one has to be careful when dealing with hemolyzed blood I am sure although I have never worked on leukocytes that they also contain a little thromboplastin I think the significance of thromboplastin is minor because you can add a little thromboplastin more than you can detect in the platelet to blood and not get the chain reaction set off in hemophilic blood

Edsall I should like to know whether Dr Brinkhous still holds to the interpretation he gave of his experiments namely that plasma of the hemophilic patient is deficient in a factor present in the normal human being which can act on platelets to release thromboplastin from them

Brinkhous I know of no data that would cause me to change the hypothesis that the platelet-plasma reaction results in liberation of platelet thromboplastin However I think this view has to be looked upon for what it is a hypothesis

Edsall As I understand your interpretation the hemophilic platelets were essentially normal The difference between the hemophilic and normal was in the plasma factor

Seegers Dr Quick have you ever tried to prepare thromboplastinogen by fractionation of plasma?

Quick No

Jaques Macfarlane [*J Clin Path* 1 *113 (1948)*] suggests that the platelets because of contact action release a lipoid factor and that the plasma proteins through contact action release a protein factor Together these two factors constitute the thromboplastin This is another interrelation of the relationship between platelets plasma and thromboplastin

Knisely It has been stated in the literature and Dr Quick brought it up again that thrombi can exist which consist only of platelets This is put in to see if we can get some sense out of the literature because I would like to follow that through How carefully were such thrombi cut and stained to be sure that only platelets were present or that there were platelets plus gluey material between them and white cells there or fibrin? Have we got somebody applying one name to something which was a mixture?

Quick Dr Zucker felt very strongly that there was no fibrin present to combine and unite the platelets She stressed the fact that these platelet thrombi were fairly pure consisting only of agglutinated platelets nothing else

I might add one more view that I have held for some time namely that hemostasis can be achieved fairly well without fibrinogen The fibrin formation is a third line of defense Children without fibrinogen have a remarkably good hemostatic mechanism for they get along much better than a hemophilic. It may well be that the fibrin formation is a late evolutionary development introduced when the blood pressure became so high in the arterial system that the more primitive mechanisms were not adequate

Wright Are there other comments?

Brinkhous In connection with the vasoconstrictor action of platelets I wonder if anyone has made platelet analyses for the crystalline vasoconstrictor substance serotonin (M M Rapport, A A Green and I H Page, [*Science* 108, *329 (1948)*] It appears entirely possible that this substance found in serum could have been liberated from the platelets

Flynn Dr Rapport brought some of his serotonin to us Our preliminary tests did not reveal it had any significant role as far as *in vitro* blood clotting is concerned

Edsall Dr Quick s scheme, I believe, involved the agglutination of platelets by the action of thrombin did it not?

Quick We generally assume that platelets agglutinate before they disintegrate but that may not be entirely true Thus from the work which Dr Barker presented it appears that changes occur in the individual platelet before it undergoes lysis Just how the platelet product is liberated into the plasma is a subject that requires further study I have made only a few microscopic studies

Edsall I thought that was part of the scheme I wondered if anyone had tried adding thrombin highly active thrombin to washed platelets and observed whether agglutination took place

STUDIES WITH A NEW SYNTHETIC ANTICOAGULANT

C W SORENSON and I S WRIGHT

Department of Medicine Cornell University Medical College

In the past year one of the projects our laboratory has been concerned with is the clinical testing of a synthetic anticoagulant called Paritol* The material is a polysulfuric ester derivative of alginic acid obtained as a water soluble sodium salt which is stable at room temperature It has been used intravenously in 1% 5% and 10% solutions

Paritol has been used 22 times in 10 patients in dosages ranging from 1 mg/kg to 10 mg/kg The effective dose required to prolong the Lee White clotting time two to three times the control time appears to be 5 to 10 mg/kg but there is some variation in the effect so that a dose of 5 mg/kg has been observed to produce a clotting time of 65 minutes one half hour after administration (control time 10 minutes) This dose requirement appears to be something in the order of ten times that of the heparin needed to produce the same maximum clotting times The duration of action of 5 to 10 mg/kg has been observed to be from 1 5 hours to 8 hours but the curves suggest that the action generally may be more prolonged than that of heparin given intravenously A slight prolongation of the prothrombin time has been observed lasting about 2 hours (Fig 26)

Immediate reactions to the slow intravenous injection of Paritol have been observed in 3 patients In one instance this was manifested by nausea vomiting abdominal cramps defecation oppression over the chest pallor sweating bradycardia and fall in blood pressure to imperceptible levels These symptoms and signs responded to prompt administration of epinephrine This patient had a history of similar reactions to intravenous papaverine and subsequently had a reaction to intravenous magnesium sulfate used in doing a circulation time (Fig 27)

One of the other reactions noted was a flushing of the face feeling of epigastric fullness with belching and a 16 mm fall in the diastolic blood pressure These manifestations disappeared

*Supplied through the courtesy of Wyeth Inc

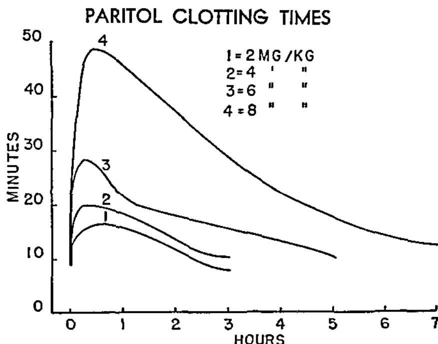


FIG 26

without medication when the injection of Paritol was discontinued

The third instance of reaction was that of a flushing of the face unassociated with change in blood pressure or pulse which disappeared while the Paritol was being continued

No toxic effect of Paritol has been observed on the blood cells, kidneys, or liver

DISCUSSION

Link How often have you given this drug to the same individual?

Wright I think some have had it four times

Link Dr Best may know more about this than I do but I personally take a dim view of the sulfuric acid esters of the various carbohydrates As a matter of fact, some years back I have forgotten whether it was 1940 or 1939 we made an excursion in that direction and thought for a while we might have something of promise However, the activities of the sulfuric acid esters of

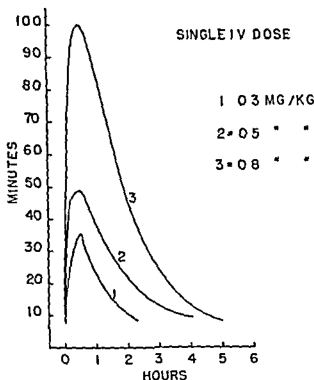


FIG 27

certain carbohydrates on drawn blood was never higher than about 1/20 that of the best heparin The activity in the cat as tested at the Abbott laboratories was about the same Furthermore a paper appeared by one of Astrup s associates which discouraged us In this paper it was pointed out that when synthetic sulfuric acid esters of any of the carbohydrates—and they studied quite a range of them—are administered to experimental animals there is a tendency for the platelets to be agglutinated with a resultant infarction of the lungs and kidneys I have seen no papers to refute the work of Astrup I have had occasion in a very cursory way to have this point checked on those products which we had made The same criticisms can be very definitely leveled against anything we had One of our products was very closely related to your

product, Dr Wright I am wondering whether or not there has been careful toxological study done from the standpoint of infarction

Wright I think your point is important Toxicological studies have been unrevealing so far I will be glad if we can obtain your reference

Link His paper came out during the war We did not get it until about 1946

Seegers In Acta Physiologica Scandinavica about 1944 by Astrup and Piper and Piper alone

Link If I recall correctly, they reported tests showing the precipitating action on fibrinogen solutions hence it acts on fibrinogen as well as on platelets When Astrup was at my laboratory last summer, I gave him the best product I had I have not heard from his as yet, but I would be surprised if it did not do the same thing as the compounds studied previously

Knisely The easiest way to test this is to stick a microscope at the side of the eye sit there watch and see what happens

Wright If there is any sludging it might not show According to Dr Link s interpretation it might not show on the first

Link After the fourth, fifth and sixth As a matter of fact, you seem to have read that paper more recently Elimination of the material was the problem Once it got caught in the lungs or kidney it stayed in there for months

Seegers There are other papers besides that also I happen to have reviewed the literature on that a couple of months ago You can collect about eight or nine papers Most of them are in the German Three of them are by Astrup and you can also get a cross reference from Dr Jorpes monograph

Knisely Can I suggest the method necessary for this? If you are going to get infarction you have got to have masses big enough to plug the vessels You should be able to pick up much smaller things happening through the blood

Wright By means of a slit lamp?

Knisely Yes

Wright We have been looking at sludging with the slit lamp in a few patients

Jaques A part of that problem is the same as that encountered with isinglass and polyvinyl alcohol and similar substances While it is true these substances are used in much lower concentration

much lower dosage level you have the same problem of accumulation of the substances in the liver and elsewhere a very long period of time is required for their elimination if they are ever eliminated

Best The problem of agglutination of platelets has arisen with respect to heparin Dr Copley and many other people have seen this clumping As I said once before at this meeting it seems unlikely that this is a physiological action of heparin although of course it might be Dr Fidlar has been studying it in our laboratory with Dr Charles who is making heparin in the Connaught Laboratories and they have had some success in preparing a product which does not possess this property to nearly the same degree as some of the commercial samples

Wright I have not presented this material with the conviction that it represents improvement over the available anticoagulants or that it is final in any sense of the word in its own right but simply to suggest that we have another tool that may be worthy of study to help us in assessing this problem of anticoagulant action

Dr Olwin did you have something to ask?

Olwin One question which is probably premature—is there any antidrug for the Paritol?

Wright We believe that the best antidote would be fresh blood transfusion but so far we have not had any evidence of bleeding

Best Have you tried protamine?

Wright No

Olwin Clinically the question of an antagonist is important in that one is likely to find himself in a situation where he has need for it

Wright We have great difficulty getting protamine

Best Isn t that available commercially for the neutralization of heparin? I thought it was

Link You have available for heparin both toluidine blue or protamine If you cannot get the one the other is available I am thinking of some real necessity

Wright We have not encountered the necessity We antici pated transfusion if necessary, but we have been using small doses in trying to control the action of this substance However as you see, two doses gave rise to levels which might have been within the range of risk

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Appendix

The high quality of the lantern slides used by Dr Seegers at the first conference on Blood Clotting and Allied Problems led to a request by the participants for the detail of their preparation

PREPARATION AND USE OF COLORED AND BLACK BACKGROUND LANTERN SLIDES

WALTER H SEEGERS EVELYN ERICKSON
and CHARLES A PICKARD

Wayne University College of Medicine

Lantern slides with a black background and colored lines have a number of advantages When they are projected there is less glare than with the conventional slides The material can be re ferred to specifically by mentioning the color of a curve rather than by use of a pointer This enables the lecturer to face his audience without intermittently turning to the screen If he wishes to use a manuscript that can be done very effectively

Lantern slides to be useful must be legible and therefore simple This sounds like an obvious statement but it is surprising that only a small percentage of slides shown at meetings and in classrooms possess that quality And a slide is not legible if it can be read only by the persons sitting in the first few rows or if it is merely serving as notes for the speaker

The material should be arranged in a shape that is in the proportion of a lantern slide A slide actually measures 3¼ x 4 An easy way to keep this proportion is to draw a diagonal through a rectangle the actual size of a lantern slide extend lines along the horizontal and vertical base lines until they encompass the material (field on which curves are plotted coordinate numbers and labels of ordinate and abscissa) that has been roughly blocked out and then cause the remaining two sides of the rectangle to meet somewhere on the diagonal line The width then measured will determine the amount of reduction and the size of lettering An illustration of this is given (Fig 28)

Because of the great variety of lenses in lantern slide pro jectors used it is safe as a rule to plan all lettering to be 2 mm high *on the slide* Therefore if the width of the material as laid out occupies 12 the reduction will be 2/3 (in other words the slide will be 1/3 the size of the copy) and the lettering should be 6 mm high And of course if you find when you lay out your lettering with 6 mm guide lines that you cannot keep the copy within 12 you will have to rearrange your copy and this may necessitate change in words

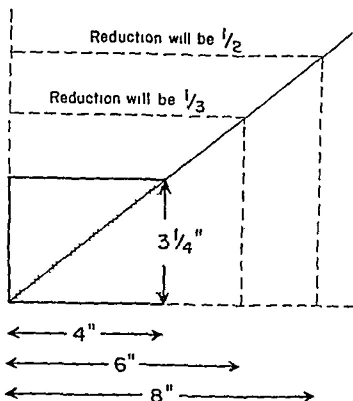


FIG 28

If the lettering is to be done freehand particular attention must be paid to keep the strokes heavy enough and clean enough to stand the amount of reduction We find that the LeRoy lettering sets (Keuffel and Esser Co 127 Fulton Street New York, and branches Chicago St Louis, San Francisco Montreal, Detroit and Los Angeles) are most satisfactory Templates No 140 (3 mm), No 200 (4½ mm) No 240 (6 mm) No 290 (7 mm), and No 350 (9 mm) provide for most situations The LeRoy pens may be used with the templates in the following combinations

1 If copy measures 6 x 4⅞" the reduction is ⅓ Use template No 140 Pen 1 or 2 or template No 200 Pen 2 or 3

2 If copy measures 8 x 6½, the reduction is ½ Use template No 200, Pen 2 or 3, or template 240 Pen 2 or 3 or 4

3 If copy measures 12" x 9¾ the reduction is ⅔ Use template No 240 Pen 2 or 3 or 4 or template No 290 Pen 3 or 4 or 5 or template No 350 Pen 3 or 4 or 5

The following are actual size samples of the above mentioned combinations of templates and pens

Template 140, PEN 1

Template 140, PEN 2

Template 200, PEN 2

Template 200, PEN 3

Template 240, PEN 2

Template 240, PEN 3

Template 240, PEN 4

Template 290, PEN 3

Template 290, PEN 4

Template 290, PEN 5

Template 350, PEN 3

Templ. 350, PEN 4

Templ. 350, PEN 5

FIG 29

The widths of curves are often too light to be as effective as possible Lining pens are often more feasible to use than ruling pens If slides are to be colored this care is particularly necessary for if color is to show across a room, the line on the negative slide should have a substantial width

The first step in the photographic process is to copy the chart on 5 x 7 contrast process orthochromatic film Exposure and focusing are extremely critical One should particularly avoid overexposure The negative is developed in Kodak D 11 for 5 minutes at 68 F A correctly exposed negative will have a dark background with clear transparent lines and no filling in of a thin line After processing all pin holes and blemishes are spotted with opaque black or opaque red, the latter being preferred This material is obtained from the Eastern Kodak Company An inter mediate is then made on a Kodak anti abrasion contrast lantern slide plate This intermediate is printed by projection We use an Eastman 5 x 7 Auto focus enlarger which has a long enough bellows to permit the projection of an image smaller than the negative It is again necessary to utilize accurate exposure This will give a positive in which the contrast is very high and in which there are but two tone values opaque and clear In our procedure, we give sufficient exposure to permit the background to supply veil and in this way strong blacks are obtained If the exposure is correct the slide can be developed for 3½ minutes at 68 F in Dektol, or D 72 (Eastman Kodak) The veil due to the desired overexposure is now cleared in Farmer s reducer This treatment results in the production of a brilliant positive lantern slide which is used as an intermediate to print the final negative slide

The final negative slide is also printed by projection in a similar manner to the positive intermediate The projected image for the positive slide is framed for the proper lantern slide mat White paper is sandwiched between two lantern slide cover glasses The lantern slide mat is also placed between the two cover glasses just as one would do before final binding Usually it is wise to select a wide mat because most projection lanterns are run by amateurs and the image is frequently too large for the average screen If a wide mat is used, this possibility is almost always eliminated As soon as the image has been focused sharply the final lantern slide plate is placed exactly in position and will be in sharp focus because the emulsion will be exactly where the white paper was placed for focusing The exposure of the image on a

contrast lantern slide is adjusted so that 3½ minutes development at 68 F in Dektol results in a slide with an intense black back ground and clear transparent lines

The materials needed to color the negative slides are a good small paint brush (Winsor and Newton finest sable hair Series 7 No 1) a booklet of transparent water color paper leaflets (Eastman), (Nicholson Peerless Transparent Water Colors Peerless Color Laboratories Rochester N Y) and a miniature muffin tin style of palette secured from any art store A small piece of color paper 1" x ½" is cut from book and is placed in one section of the muffin tin Water is added with eye-dropper—just 3 or 4 drops With the relatively dry brush the color is applied to the emulsion surface of the slide We have found that yellow red and green—in that order—are the most satisfactory colors If it is necessary to use a fourth color blue it is advisable that it be applied as a very light tone—otherwise there is a danger that it will become opaque

After coloring pin holes and other blemishes are specked with India ink The slide is then ready for binding